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LYSIS OF RED BLOOD CELLS BY TISSUE SLICES.

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THE discovery by Maegraith, Martin and Findlay (1943*a*) of an erythrocytolytic enzyme in animal tissues, which is counterbalanced by an inhibitory factor in serum, seemed to offer a simple explanation for the mechanism of normal blood destruction. Moreover, a reduction of this serum inhibition should result in pathologically increased red cell destruction ; that this can actually occur was demonstrated in a few cases of blackwater fever (Maegraith, Findlay and Martin, 1943*b*).

It was with this possibility in mind that we intended to apply the method of Maegraith and co-workers to a phenomenon which is particularly frequent among the oriental-Jewish section of the Palestinian population. This is the peculiar hypersensitivity of certain individuals towards sulphonamides, fava beans, certain chemicals, toxins in typhoid and other infections, resulting in acute severe haemolytic anaemia ; one is tempted to speak about a haemolytic constitution, to use a phrase coined by Gänsslen (1925).

Early in this study it became apparent that some of the claims of Maegraith and his co-workers could not be substantiated. This led us to a thorough re-investigation, which is the subject of the following report. In view of the evidence presented below we are in doubt whether the *in vitro* lysis of red cells by tissue slices bears any relationship to blood destruction *in vivo*.

METHODS.

The technique of Maegraith *et al.* was strictly followed, with two exceptions : (1) citrated instead of defibrinated blood was used, after preliminary experiments had shown that identical results were obtained with both methods ; (2) the washed red cell concentration of the systems was adjusted to 2·5 per cent instead of 5 per cent, since with the higher concentration complete lysis was seldom obtained in less than three days. All other procedures, including aseptic technique, bleeding of the smaller animals (mostly rats and guinea-pigs), preparation of tissue slices about 4 mm. square and 2 mm. thick, washed three times for 20 minutes at 4°C., use of 0·85 per cent saline in phosphate buffer of pH 7·1 for all washings and dilutions were essentially those described by the above-mentioned authors.

The first readings were usually taken after 17–19 hours incubation at 37° C., thereafter at about 21, 24, 42 and 48 hours. Before each reading the tubes were thoroughly shaken, then centrifuged for two minutes at low speed, and the degree of lysis was judged by the colour of the supernatant solution. Before further incubation the tubes were reshaken ; the whole reading procedure took about 20 minutes. During

the first part of this study, results were classified as follows: No lysis (NL), slight lysis (SL), partial lysis (PL) and total lysis (L). Later, a simple semi-quantitative procedure was adopted, in which the colour of the supernatant liquid was compared with a set of standard haemoglobin solutions, prepared by haemolysing various amounts of the particular red cell suspension used in the experiment. In this case, results were expressed as per cent lysis, and are of course to be considered only as rough approximations. If extracts were used, these were always diluted so that 1 c.c. of extract corresponded to one slice of tissue of standard size. A total of 80 experiments was carried out, using 26 guinea-pigs, 19 rats and a number of animals of other species.

RESULTS.

I. *Lytic Action of Tissue Slices.*

The principal finding of Maegraith *et al.*, that washed tissue slices are able to haemolyse washed red cells within 24–48 hours at 37° C. was easily confirmed in our experiments. Marked differences exist, however, not only between different tissues from the same animal, but also between single slices of the same origin, so that many experiments had to be made in duplicate or triplicate. These findings are illustrated in Table I, which at the same time gives the kinetics of the lytic process in some typical

TABLE I.—*Lytic Action of Various Tissues.*

System: Guinea-pig tissues/guinea-pig red cells.

Exp. No.	Tissue.	Percentage lysis after hours						
		18	21	23	26	42	45	1
46	Liver	6	..	16	25	..	..	..
46	„	10	..	20	35	100	..	..
46	„ half size	15	..	25	40	100	..	..
46	None	0	..	0	0	5	..	..
56	Liver	..	3	..	5	..	80	..
56	„	..	13	..	23	..	80	..
56	Kidney	..	6	..	10	..	50	60
56	„	..	12	..	25	..	80	..
56	Lung	..	0	..	3	..	12	20
56	„	..	2	..	4	..	90	..
56	Heart	..	2	..	4	..	10	10
56	„	..	2	..	6	..	25	40

experiments. The lytic power of the different organs decreases in the order liver, kidney, lung, heart, the latter two being far inferior to liver. For this reason, liver was used in most of our experiments, to shorten as far as possible the time required for complete lysis, which even with this organ averaged 47 hours. Varying the size of the liver slices within the range $\pm$ 100 per cent had little influence, but liver brei, in three tests, showed more rapid lysis than the standard slices. With very thin slices, as used in Warburg's technique, lysis proceeded about normally.

Heating the slices for 10 minutes at 80° C. resulted in complete loss of lytic power in rat and guinea-pig tissues, in accordance with Maegraith.

II. *Inhibition of Lysis.*

In spite of many attempts to verify Maegraith's claim that serum, cyanide and mercuric chloride are powerful inhibitors of the lytic process, we were unable to obtain clear-cut inhibition. In some 20 experiments with guinea-pig and rat liver and homogenous and foreign sera in various dilutions, we never encountered inhibition, and in only a few cases did lysis proceed at a slower rate than in the controls without serum.

Potassium cyanide in amounts ranging from 5 to 500 μg. was without any influence on guinea-pig and rat liver, and mercuric chloride in amounts between 40 and 800 μg. had a clearly accelerating effect on the lytic action of these tissues. A few trials with other organs (lung, heart) gave no better results, with the exception of a single experiment in which the lysis by heart tissue was inhibited by addition of 0·01 c.c. of guinea-pig serum. Some of these experiments are tabulated in Table II.

TABLE II.—*Influence of Various Agents on Lysis.*

System : Guinea-pig tissues/guinea-pig red cells.

Exp. No.	Tissue.	Addition.	Percentage lysis after hours				
			18	21	26	42	45
56	Liver	..	..	3	5	..	80
56	„	..	..	15	23	..	80
56	„	0·01 c.c. human serum	..	18	28	..	70
56	„	0·02 c.c. guinea-pig serum	..	3	8	..	80
56	Kidney	..	..	12	25	..	80
56	„	0·02 c.c. guinea-pig serum	..	10	22	..	90
56	„	75 μg. KCN	..	5	20	..	90
58	Liver	..	12	..	35	100	..
58	„	..	25	..	80	100	..
58	„	0·05 c.c. guinea-pig serum	23	..	60	100	..
58	„	75 μg. KCN	23	..	60	100	..
58	Lung	..	25	..	40	70	..
58	„	..	5	..	8	15	..
58	„	0·05 c.c. guinea-pig serum	5	..	8	15	..
58	„	0·01 c.c. „ „	90	..	..	..	..

While as a rule bacteriological examinations for sterility were not performed, we decided to check a few experiments in which serum had been added.* In Exp. No. 56 (Table II) the tubes were found to be contaminated with an air-born coccus, which proved to be non-haemolytic on blood-agar culture. In Exp. No. 58 the tubes were sterile. Since the results are essentially the same in both cases, we feel that lack of sterility is not responsible for the absence of serum inhibition in our systems.

III. *Species Specificity of the Lytic Agent.*

One of the most striking features of Maegraith's erythrocytase appears to be the species specificity claimed to be present in human, monkey and guinea-pig tissues. We have extended this series to three more species, viz., the rat, rabbit and mouse, using up to 9 kinds of heterogenous washed red cells, and have found that species specificity is not the rule (Table III). The only exception appears to be the guinea-pig,

TABLE III.—*Species Specificity of Tissue Lysis.*

Liver tissue. Results after 24–48 hours. No. of experiments exceeding one indicated in brackets.

Red cells.	Guinea-pig.	Rat.	Man.	Rabbit.	Mouse.
Man	SL (5)	PL (2)	L	PL	L
Monkey	NL	PL (2)	SL	L	PL
Calf	NL	..	SL	PL	PL
Guinea-pig	L (15)	L (5)	L	L	..
Rat	PL (3)	L (13)	L	L	L
Sheep	NL	PL (2)	SL	..	..
Rabbit	..	..	..	L	PL
Chicken	SL (2)	SL	L	L	..

* Kindly carried out by Mr. Stuezinski.

whose tissues are lytic only upon guinea-pig and to a lesser extent upon rat erythrocytes. With the other species tested, irregular differences in lytic action upon various red cells could be observed, and sometimes foreign blood was actually more rapidly haemolysed than homogenous. In the meantime a paper has appeared by Ponder (1944), in which the species specificity of the lytic agent could also not be confirmed.

IV. *Inhibition of Spontaneous Lysis by Tissue Slices.*

While testing the species specificity of the lytic process a peculiar phenomenon was revealed which, as far as we know, has not yet been described before. In the control experiments the red cells of some species, chiefly dog and mouse, sometimes rabbit, and very occasionally rat, underwent spontaneous lysis at 37° C. This process was either completely inhibited or else markedly slowed in the presence of tissue slices, of the same or a foreign species (Table IV). Thus the somewhat paradoxical situation

TABLE IV.—*Inhibition of Spontaneous Lysis.*

Exp. No.	Red cells.	Tissue.	After hours	
			24	42
17	Rabbit	..	SL	PL
17	„	Guinea-pig liver	SL	SL
34	Mouse	..	..	PL
34	„	Rat liver	..	SL
37	Dog	..	L	L
37	„	Dog liver	NL	SL
37	„	Rabbit liver	SL	PL
40	Mouse	..	PL	PL
40	„	Rat heart	NL	PL
42	Dog	..	..	L
42	„	Dog heart	..	NL

could arise in which two slices of the same mouse liver, acting on two different samples of rat erythrocytes, would behave antagonistically. Where the rat red cell control remained intact, the addition of mouse liver would cause complete lysis within 42 hours; when, for some unknown reason, the second rat control underwent spontaneous lysis, this could be abolished by the presence of mouse liver.

This observation is the more difficult to explain as the mechanism of the spontaneous lysis of some red cells at 37° C.—a fact which has long been known—has not yet been elucidated. Some recent investigators (Ham and Castle, 1940*a*, *b*; Tsai, Chen and Chiu, 1943) believe that accumulation of osmotically active metabolites within the stagnant cells might be responsible. It is hard to understand how the presence of tissue slices could influence this process. Another possibility would be that tissues contain at the same time both lytic and inhibiting agents, the former being neutralized by some red cell compounds set free during spontaneous haemolysis. To prove this point, we have added red cell haemolysates to some of our systems, without influence on the lytic process.

V. *Influence of Pre-Incubation on Lysis.*

Our findings as reported so far are not inconsistent with the view that the chief factor responsible for the lytic action of tissue slices, in our systems, is autolysis. If this is true, pre-incubation of the slices before bringing them into contact with the red cells should greatly enhance the lysis of the latter. In Table V 16 experiments are summarized in which guinea-pig liver had been kept for various periods, from one to

TABLE V.—*Lytic Action of Pre-Incubated Slices.*

System : Guinea-pig red cells/guinea-pig liver.

Exp. No.	Treatment of slices.	Percentage lysis after hours					
		6	18	22	25	40	44
54	Stored at 4° C.	..	15	..	40	..	100
54	Incubated at 37° C.	..	60	..	80	..	100
50	Fresh slice	..	30	50	60	..	100
50	„ „	..	24	40	55	..	100
50	Stored at 4° C.	4	..	20	45	..	90
50	Incubated at 37° C.	16	..	95	100	..	..

three days, at 37° C. in phosphate buffer, and then compared with other slices which had been kept for the same period at 4° C. In each experiment lysis was much more rapid with the pre-incubated samples. It was observed, however, that some of the 4° samples had a slower lytic action than normal fresh slices. Even if this fact is taken into account, the superiority of the pre-incubated slices remains evident, as judged by the average times required for complete lysis. These are : 47 hours for normal fresh slices (range 24–70, 20 experiments), 66 hours for slices kept at 4° C. (18–100, 18 experiments), and 35 hours for slices kept at 37° C. (16–60, 16 experiments). These findings are in contrast to those of Maegraith who found no difference between normal and pre-incubated tissues.

Storage of red cell suspensions of various species for several days at 4° C. was without influence on their behaviour in the lytic systems.

VI. *Influence of Mechanical Factors on Lysis.*

During attempts to enhance the lysis of red cells we were surprised to observe that shaking the system resulted in marked inhibition of the lytic process. This is shown in Table VI and suggests that besides autolysis a second factor is in operation.

TABLE VI.—*Influence of Shaking on Lysis.*

System : Guinea-pig red cells/guinea-pig liver.

Exp. No.	Time of shaking	Percentage lysis after hours			
		17	22	25	42
49	First 24 hours	..	..	10	12*
49	„ „	..	..	4	25
49	„ „	..	..	4	15
49	No shaking	12	20	30	80
49	„	10	18	28	80
55	First 18 hours	0	..	8	20
55	No shaking	10	..	20	85
58	First 25 hours	3	..	12	70
58	„ „	3	..	20	80
58	Hours 18–25	20	..	55	100
58	No shaking	12	..	35	100
58	„	25	..	80	100

* Red cells alone.

namely stasis. Its influence was most conspicuous during the first 18 hours of incubation, which may conveniently be termed the induction period of the lytic process. Thereafter, shaking was of little influence on the course of lysis. On the other hand, attempts to intensify lysis by prolonged stasis for 24 or 40 hours, instead of the usual 17–22 hours, were unsuccessful, and normal results were obtained. Systems with

pre-incubated liver or lytic liver extracts (see below) were not influenced by shaking; apparently the much higher lysin content of these systems suffices to induce lysis even without stasis.

VII. *Lytic Action of Tissue Extracts.*

In order to obtain further proof of the non-enzymatic nature of the lytic agent, attempts were made to extract the latter by various methods. We are aware that no reliable conclusions can be drawn from such experiments since the fact that a lytic substance is present in a tissue extract does not necessarily mean that the same substance is responsible for the lytic effect of the tissue itself. Moreover, as Ponder (1944) recently pointed out, tissues contain a great number of potentially lytic and inhibitory substances, the properties of which may be altered by the extraction process, a fact which makes identification of a particular lysin even more difficult.

The following extracts were made under aseptic conditions from washed tissue slices: (*a*) Water Extract, Fresh. The slices are ground with glass powder and saline-phosphate buffer pH 7·1 centrifuged, and the extract made up to volume. (*b*) Water Extract, Old. Prepared like (*a*), but using pre-incubated slices. (*c*) Alcohol Extract, Fresh, Direct. Fresh slices are extracted with ordinary alcohol, the extracts evaporated *in vacuo* and the residue taken up in buffered saline. (*d*) Alcohol Extract, Old, Direct. Prepared like (*c*), but using pre-incubated slices. (*e*) Alcohol Extract, Fresh, Indirect. The extracts described under (*a*) are treated with 10 volumes of alcohol for some minutes at 50°, filtered, the filtrate evaporated *in vacuo* and the residue taken up in buffered saline. (*f*) Alcohol Extract, Old, Indirect. Prepared like (*e*), but using the water extracts described under (*b*). (*g*) Lysolecithin Extract, Fresh. This is prepared according to the directions of Singer (1940) for the isolation of lysolecithin from serum, replacing the latter by tissue slices or their aqueous extracts. After treatment with alcohol and filtration, the filtrate is evaporated, and the residue suspended in ether and left overnight at 4° C.; the ether is then discarded and the ether-insoluble residue taken up in buffered saline. (*h*) Lysolecithin Extract, Old. Prepared like (*g*), but using pre-incubated slices or their extracts. (*i*) Medium. This is not an extract, but the buffered saline in which tissue slices had been incubated for one or two days.

It was observed that all extracts could be kept on ice for some days without losing their activity. With the exception of extracts (*e*) the results were quite consistent and show (Table VII): (1) Fresh water extracts or medium are without any lytic

TABLE VII.—*Lytic Action of Various Extracts of Guinea-pig Liver.*

Figures in brackets indicate number of experiments.

Type of extract.	Reading after hours	
	24	42
a. Water, fresh	NL (8)	L (7)
b. Water, old	L (9), SL (1)	L (1)
c. Alcohol, fresh, direct	SL (2)	L (1) SL (1)
d. Alcohol, old, direct	L (1)	..
e. Alcohol, fresh, indirect	NL (2), L (2)	L (3)
f. Alcohol, old, indirect	L (3), SL (1)	L (1)
g. Lysolecithin, fresh	NL (3)	SL (1)
h. Lysolecithin, old	NL (2)	SL (1), L (1)
i. Medium	NL (7)	NL (6), PL (1)

action during the first day of incubation or longer, and only the former become lytic after this interval. (2) The same is true with fresh direct alcohol extracts, whereas

type (*e*) is sometimes lytic. (3) The water or alcohol extracts of pre-incubated slices are strongly lytic, more in fact than the pre-incubated slices themselves. Complete lysis always occurred within 20 hours, and the haemoglobin was usually found deposited as crystals with the typical shape of guinea-pig (or rat) haemoglobin. (4) Lysolecithin does not seem to be present in fresh or incubated tissue slices.

DISCUSSION.

The finding that tissues and their extracts may be lytic to erythrocytes is not new; there are many references to the matter in the literature of the first decade of this century, chiefly concerned with the normal and pathological spleen. Ponder (1944) has already recalled the early experiments of Weil (1907). Among the more recent authors, Katuyama (1939) has tested extracts of perfused rabbit organs upon dog erythrocytes and found them lytic only when the tissues had undergone autolysis, which is in accordance with our results.

The chief points in favour of a lytic enzyme, in the work of Maegraith and his associates, are the species specificity, and the inhibition by typical enzyme poisons and by heat. We have been able to reproduce only the last finding. On the other hand, assuming the presence of a true erythrocytase in the tissue slices, it can hardly be understood why (*a*) a period of about 18 hours should be necessary to induce lysis; (*b*) pre-incubation should greatly increase the activity of this enzyme; (*c*) shaking the system should merely abolish its action.

We think that these facts are more easily explained by the assumption that lytic substances, e.g. fatty acids, moderately soluble in water, are liberated from tissue slices during autolysis and penetrate the membranes of red cells which have had sufficiently long contact with the slices. If this contact period is shortened by shaking the amount of lysin absorbed by the cells is insufficient to induce lysis. (An alternative explanation would be that only red cells which have been rendered more fragile by prolonged stasis at 37° C. (Fahraeus, Bergenheim, 1936; Fahraeus, 1939; Ham and Castle, 1940*a*, *b*) are haemolysed by the lytic agent). After pre-incubation, or in extracts of pre-incubated tissues, sufficient lytic material is available to induce lysis even without stasis. The fact that this material is alcohol-ether soluble proves its non-enzymatic nature, assuming its identity with the original lysin in the slices.

The incubation period is thus dependent mainly on the degree of autolysis, and is consequently shortened with tissues which have been pre-incubated.

Ponder (1944) in his re-investigation of the claims of Maegraith *et al.* has advanced the view that the enzyme responsible for the liberation of the lytic substance might be lysolecithinase and the agent itself lysolecithin, which would thus appear to be much more widely distributed than formerly assumed. As reported, we have been unable to extract lysolecithin from fresh and even autolysed tissues; moreover, addition of lecithin to our systems did not increase lysis.

A real difficulty lies in the fact that red cells of foreign species are sometimes attacked much slower than those of homogenous blood. If differences in fragility were responsible, the same order of fragility should be expected with all tissues, which clearly is not the case. Our present knowledge of the nature of the lytic factor in tissues undergoing autolysis is too restricted to allow of a satisfactory explanation.

SUMMARY AND CONCLUSIONS.

A re-investigation of the experiments of Maegraith *et al.* concerning the lytic action of tissue slices gave the following results:

1. Washed tissue slices are able to haemolyse washed red cells. Lysis begins after an induction period of about 18 hours, and is complete in 2–3 days, depending on the tissue used.

2. Shaking the system during the induction period results in marked inhibition of the lytic process.

3. Pre-incubation of the tissues shortens the induction period and results in more rapid lysis.

4. Serum, potassium cyanide and mercuric chloride in various dilutions do not inhibit lysis ; this effect may be brought about by heating the tissues to 80° C.

5. The spontaneous lysis of the red cells of some species is inhibited or slowed by the presence of homogenous or foreign tissues.

6. Guinea-pig liver haemolyses guinea-pig and rat erythrocytes much more rapidly than those of other species. With other tissues, irregular differences are observed with various red cells.

7. While water and alcohol extracts of pre-incubated tissues are strongly lytic, extracts from fresh tissues become lytic only after 24 or more hours. Lysolecithin could not be detected in fresh or incubated tissues.

These findings indicate that the lytic phenomenon studied depends mainly on two factors, autolysis and stasis, and is not connected with normal or pathological blood destruction.

REFERENCES.

BERGENHEIM, F., AND FAHRAEUS, R.—(1936) *Z. ges. exp. Med.*, **97**, 555.

FAHREAUS, R.—(1939) *Lancet*, ii, 630.

GÄNSSLEN, M.—(1925) *Dtsch. Arch. klin. Med.*, **146**, 25.

HAM, T. H., AND CASTLE, W. B.—(1940*a*) *Trans. Ass. Amer. Physicians*, **55**, 127.—(1940*b*) *Proc. Amer. Philosoph. Soc.*, **82**, 411.

KATUYAMA, S.—(1939) *Okayama Igak. Zas.*, **51**, 1658, ref. *Chem. Abstr.*, **37**, 1174.

MAEGRAITH, B. G., MARTIN, N. H., AND FINDLAY, G. M.—(1943*a*) *Brit. J. exp. Path.*, **24**, 58.

MAEGRAITH, B. G., FINDLAY, G. M., AND MARTIN, N. H.—(1943*b*) *Lancet*, i, 573.

PONDER, E.—(1944) *J. gen. Physiol.*, **27**, 483.

SINGER, K.—(1940) *Amer. J. med. Sci.*, **199**, 466.

TSAI, CH., CHEN, C. J., AND CHIU, K. Y.—(1943) *Amer. J. Physiol.*, **138**, 519.

WEIL, R.—(1907) *J. med. Res.*, **16**, 287.

THE ARGININE, ORNITHINE AND CARBON DIOXIDE REQUIREMENTS OF STREPTOCOCCI (LANCEFIELD GROUP D) AND THEIR RELATION TO ARGININE DIHYDROLASE ACTIVITY.

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THESE investigations were started with the intention of studying the effect of sulphanilamide on the growth and metabolism of streptococci belonging to Lancefield's Group D. It was first necessary to devise a simplified medium that would support a rapid and heavy growth of the organisms, and also to obtain some knowledge of the changes brought about in such a medium during growth as a result of the metabolism of the organisms. Group D streptococci are highly exacting in their nutritional requirements: Schuman and Farrell (1941) found that certain strains will grow in a medium containing salts, pyridoxin, pantothenic acid, riboflavin, arginine, glutamic acid, methionine (replaceable in some cases by cysteine), tryptophan, tyrosine, valine and glucose; Niven and Sherman (1944) have shown that other strains require, in addition to these factors, nicotinic acid, biotin, "folic acid," and a further eight amino acids. McIlwain (1940) found that *Strept. haemolyticus* can grow in a medium in which the arginine is replaced by ornithine. Washed suspensions of these streptococci attack only two of the common amino acids at a significant rate: arginine which is hydrolysed to ornithine and ammonium carbonate by arginine dihydrolase (Hills, 1940), and tyrosine which is decarboxylated to tyramine (Gale, 1940). The tyrosine decarboxylase is formed only when growth takes place at an acid pH (Gale, 1940), and in the presence of nicotinic acid and pyridoxin in excess of simple growth requirements (Bellamy and Gunsalus, 1944). From these studies it would appear that the organisms are nutritionally exacting towards arginine while possessing an enzyme which destroys this amino acid; the present communication deals with the relation between this enzymatic activity, the requirements of arginine and ornithine, and the changes taking place in the medium during growth of certain group D streptococci.

EXPERIMENTAL.

Organisms and Methods.

Organisms.—The greater part of this work was carried out with a non-haemolytic Group D streptococcus (ST) isolated in this Unit, and now deposited in the National Collection of Type Cultures (No. 6782). Three other strains of Group D streptococci were used: F315 and Duff being haemolytic, and *Strept. faecalis* Dunn non-haemolytic. Washed suspensions of the organisms grown for 16 hours in glucose-casein digest medium had the following arginine dihydrolase activities (Q_{CO_2}): ST, 156; F315, 28; Dunn, 2; Duff, 0·8.

Estimation of growth.—Growth was estimated turbidimetrically in a Hilger Photoelectric Absorptiometer using a neutral filter. The instrument was calibrated empirically against suspensions of the organisms, the dry weights of which were determined by drying in a steam oven to constant weight. In the diagrams the amount of growth is, in some cases, quoted directly as absorptiometer scale readings.

Arginine dihydrolase activity.—This was estimated manometrically with washed suspensions of the organisms: manometers were set up with 1·0 ml. suspension and 1·5 ml. M/5 phosphate buffer pH 6·0 in the main cup, and 0·5 ml. M/15 arginine at pH 6·0 in the side bulb. Measurements of CO_2 output were taken every 5 min. after the addition of substrate and the rate Q_{CO_2} (µl. CO_2 liberated from arginine/hr./mg. dry weight of organism) calculated from the steady gas output for the first 15 min. at 37° C.

Estimation of arginine.—The manometric estimation of certain amino acids using specific amino acid decarboxylase preparations has been described by Gale (1945). A specific preparation of arginine decarboxylase has not yet been obtained, but acetone powders of *Esch. coli* (N.C.T.C. No. 86) prepared as for the preparation of lysine decarboxylase (Gale and Epps, 1944) are specific for the decarboxylation of lysine and arginine. Suspensions of such powders, 20 mg./ml., in M/5 acetate buffer pH 5·2 can be used for the estimation of arginine in the absence of lysine. Control estimations on standard arginine solutions gave 96 per cent theoretical CO_2 evolution under the usual conditions. Ornithine was estimated as previously described (Gale, 1945).

Measurement of rate of fermentation.—Group D streptococci perform a simple *homo*-lactic fermentation with no gas production when glucose is the substrate. The rate of lactic acid formation was studied manometrically, using M/80 $NaHCO_3$ as a buffer in the presence of 5 per cent CO_2 in N_2 at 37° C. Rates of fermentation are expressed as µl. CO_2 liberated by lactic acid formed/hr./mg. dry weight of organism (Q_{CO_2}). The non-production of CO_2 as a fermentation product was confirmed.

Preparation of Basal Medium.

None of the four organisms used in this work would grow in the synthetic medium described by Schuman and Farrell (1941), and it was not possible to prepare the complex medium of Niven and Sherman (1944) on the scale required for these studies. The organisms grew well on a medium containing salts, glucose, arginine and marmite; it will be shown below that the growth varies greatly according to whether certain amino acids are supplied in a free state or as peptides, so for the basal medium it was decided to hydrolyze the marmite to split any proteins and peptides therein to amino acids. The marmite (5 per cent in *ca.* 5 N HCl) was refluxed for 22 hr., the excess HCl removed by repeated evaporation to dryness *in vacuo*, and the hydrolysate made up in solution equivalent to 10 per cent marmite and neutralized by the addition of NaOH. Precipitated humin was filtered off. In the presence of excess arginine, optimal growth was obtained with 0·1 per cent marmite in the medium; after hydrolysis the marmite no longer acts as a source of certain growth factors which must therefore be added to the medium. Some of the organisms also require the addition of cysteine for consistent growth, and the composition of the basal medium finally used is given in Table I.

The effect of glucose concentration on growth in this medium plus excess arginine was studied; optimum growth was obtained with 0·5–1·0 per cent glucose. The medium as prepared contains 1·3 mg. lysine and 0·4 mg. arginine per 100 ml. and will not support the growth of Group D streptococci with high arginine dihydrolase activity (see Fig. 1). Growth occurs rapidly in the presence of added arginine, being

TABLE I.—*Composition of Basal Medium.*

KH_2PO_4	100 mg.	Pyridoxin*	150 μg.
$MgSO_4$, $7H_2O$	70 ,,	Riboflavin*	150 ,,
NaCl	100 ,,	Ca pantothenate*	300 ,,
$(NH_4)_2HPO_4$	400 ,,	Cysteine HCl*	56 mg.
$FeSO_4$, $7H_2O$	1 ,,	Glucose*	1 g.
10 per cent. hydrolysed marmite	1 ml.	Distilled water	99 ml.

* Sterilized by filtration.

Unless otherwise stated, the medium was adjusted to pH 7·4.

complete within 9–10 hr. after inoculation, and giving a final crop of organism equivalent to 0·35–0·40 mg. dry weight/ml. medium. In those cases where ornithine was added to the medium, it was found necessary to sterilize this by filtration, as autoclaving results in the destruction of 50–60 per cent of the ornithine as estimated by *l* (+) – ornithine decarboxylase.

Organisms grown in this medium do not develop significant tyrosine decarboxylase activity. It was found difficult to maintain cultures in this medium and accordingly the organisms were kept in casein digest-glucose broth, and unless otherwise stated the basal medium was inoculated with 0·5 per cent of its volume of a 24-hr. culture in broth ; estimation of the arginine and ornithine contents of such broth cultures before and after growth show that all the arginine in the broth is converted to ornithine during growth, and consequently the amount of arginine added with the inoculum is negligible. The inoculum described amounts to *ca.* 10^6 organisms/ml. final inoculated medium.

In the following descriptions basal medium plus 0·1 per cent arginine is referred to as "arginine medium" ; basal medium plus 0·04 per cent ornithine as "ornithine medium."

Cultures were incubated at 37° C.

Arginine Requirement.

Variation with Arginine Dihydrolase Activity.

Fig. 1 shows the variation of total growth with the arginine content of the medium for four strains of Group D streptococci having different arginine dihydrolase activities. The growth of those organisms with appreciable activity is dependent upon the amount of arginine in the medium ; the growth of organism ST is not optimal until the arginine concentration reaches *ca.* 1 mg./ml. medium. Where the arginine dihydrolase activity is small growth is optimal for a correspondingly low arginine content of the medium.

Variation with pH of Medium.

For the experiments shown in Fig. 1 the medium was adjusted to pH 7·4 before inoculation and this falls to *ca.* 4·5 during growth, due to the fermentation of the glucose to lactic acid. In the case of the organism ST the utilization of arginine varies considerably with the initial pH of the medium as shown in Fig. 2. The utilization is more efficient when the initial pH of the medium is adjusted to a value greater than 7·0, as (1) the limit of acid tolerance is reached more slowly, and (2) the rate of destruction of arginine is most rapid at the optimum pH of arginine dihydrolase which lies between 5·5 and 6·0. If the fall of pH during growth is followed it is found that optimum growth is obtained when the amount of arginine in the medium is such that the limit of acid tolerance is reached before all the arginine has been decomposed.

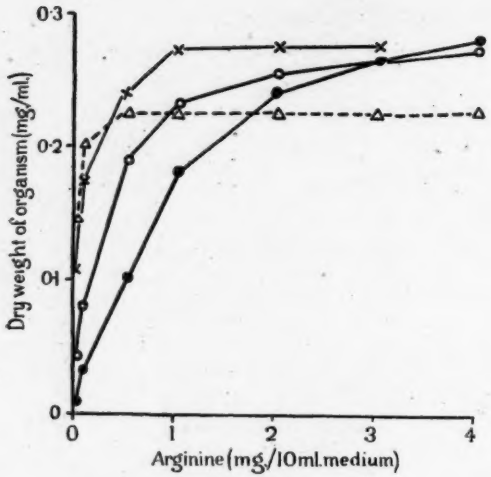

FIG. 1.

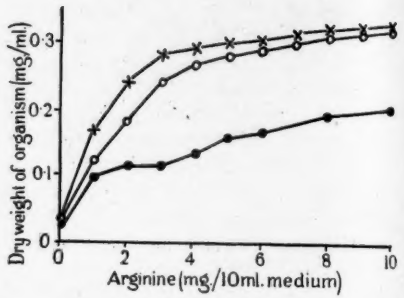

FIG. 2.

FIG. 1.—Variation of arginine requirement with arginine dihydrolase activity. Growth after 18 hr. incubation; initial pH of medium = 7·4. ——●—— Strain ST (Q_{CO_2} = 156); ——○—— F315 (Q_{CO_2} =28); ——×—— Dunn (Q_{CO_2} = 2·0); ——Δ—— Duff (Q_{CO_2} = 0·8).

FIG. 2.—Variation of arginine utilization with initial pH of medium. ——●—— pH 6·1; ——○—— pH 7·0; ——×—— pH 7·7. Growth after 16 hr. incubation.

Free or Combined Nature of Arginine.

During early work on the growth of organism ST it was found that the crop obtained in a medium containing tryptic digest of casein was twice or three times as great as that obtained in a medium containing an equivalent amount of acid hydrolysate of casein, although an excess of tryptophan and growth factors was added to the latter medium. The main difference between the two media seemed to lie in the arginine content; in the hydrolysate all the arginine was present as free amino acid, while in the digest about 60 per cent was present in some combined state from which it was liberated during growth, presumably by the proteolytic activities of the organism. The question arises whether it is more effective to supply arginine in the medium in the form of a peptide from which the free amino acid can be gradually liberated during growth than to supply it as free amino acid from the beginning. To test this point it was necessary to have an arginine-peptide which could be added to the simplified basal medium and which can be hydrolysed by the streptococcal enzymes. In view of the difficulty of synthesizing such peptides, it was decided to use a partially degraded preparation of the protamine Salmin which contains 90 per cent arginine (Taylor, 1908). The organism was unable to grow in basal medium plus pure Salmin. Salmin was dissolved in 5·5 N HCl, left to stand at 37° C. for 16 hr., and the excess HCl then removed by evaporation to dryness *in vacuo*. The resulting preparation contains no free arginine when tested with arginine decarboxylase and must consist mainly of arginine-peptides. Fig. 3 shows the growth of organism ST obtained in basal media, initial pH 7·4, to which varying amounts of free arginine or "arginine-peptide" were added. The peptide supports growth much more efficiently than the free amino acid, optimal growth requiring approximately twenty times as much free arginine as "arginine-peptide." The difference cannot be explained by the presence of other

amino acids in the Salmin preparation, as the addition of appropriate amounts of these (alanine, serine, proline) to the medium containing free arginine had no effect on the result.

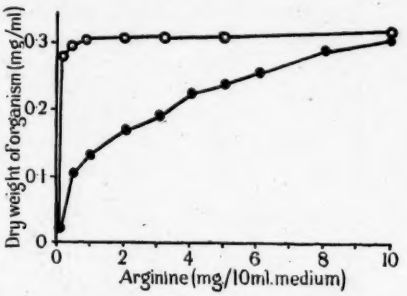

FIG. 3.—Variation of growth of organism ST with arginine content of medium. Growth after 16 hr. incubation. —●— free arginine ; —○— "arginine-peptide."

Ornithine and Carbon Dioxide Requirements.

Ornithine Concentration.

The total growth obtained in the basal medium (initial pH 7·4) with the addition of varying amounts of arginine or ornithine was compared. The response to ornithine was irregular and the crop not significantly increased by concentrations of ornithine greater than 200 μg./ml. The optimum crop with ornithine was only *ca.* 25 per cent of that obtained with arginine. Arginine can, however, be replaced to some extent by ornithine, and it is possible that ornithine is converted into arginine, in which case the irregular nature of the growth response to ornithine may be due to variations in the CO_2 concentration in the gas phase during growth.

Concentration of Carbon Dioxide.

Flasks were made up with basal medium containing 40 mg. ornithine/100 ml. and were fitted with gassing and sampling tubes. Four flasks were gassed with, respectively, CO_2-free N_2, air, 5 per cent CO_2 in N_2, and 10 per cent CO_2 in N_2. The flasks were inoculated and incubated as usual and samples removed during growth. Fig. 4 shows that the rate of growth and total crop are greatly influenced by CO_2 concentration ; it would appear that the enzyme system concerned with the assimilation of CO_2 is almost saturated by 5 per cent CO_2, as increasing the partial pressure above this has little enhancing effect on growth rate. In the culture gassed with CO_2-free N_2 there is a small growth during the first 5 hr., after which growth is negligible. To confirm that this small growth is due to residual CO_2 dissolved in the medium, the experiment was repeated with CO_2-free medium ; the medium was made up at pH 6·5, brought to boiling and then neutralized to pH 7·2 with CO_2-free NaOH, cooled in a stream of CO_2-free air, and sterilized by filtration through a Seitz filter in a CO_2-free atmosphere. The medium was filtered into two flasks each fitted with a sintered-glass bubbler, each flask inoculated with a loopful of culture taken from growth on an agar slope, and incubated one in a stream of CO_2-free N_2 and the other in a stream of 5 per cent CO_2 in N_2. Within 24 hr. the medium gassed with the CO_2 gas mixture was turbid, while the medium in CO_2-free conditions was completely clear. After 5 days,

during which no growth occurred in the CO_2-free medium, this was gassed with 5 per cent CO_2 in N_2; full growth then took place within 24 hr.

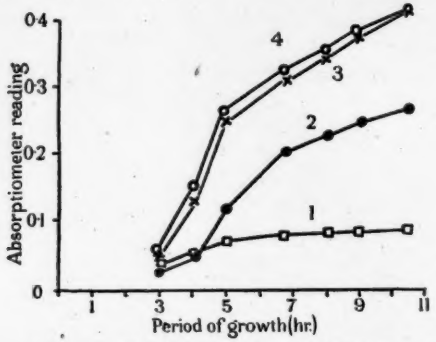

FIG. 4.—Effect of CO_2 concentration on the growth of organism ST in "ornithine medium." Medium gassed with (1) CO_2-free N_2, (2) air, (3) 5 per cent CO_2 in N_2, (4) 10 per cent CO_2 in N_2.

Carbon Dioxide Requirement.

Valley and Rettger (1926, 1927) and Gladstone, Fildes and Richardson (1935) have shown that many bacteria are unable to grow in the absence of CO_2. This can be demonstrated relatively easily when organisms will grow in a simple synthetic medium, but when a complex medium is necessary the metabolism of the inoculum often results in CO_2 production, and in such cases it is possible only to show a delay in the appearance of growth in a CO_2-free medium as opposed to normal medium. With the organism ST no growth will take place in "ornithine medium" in the absence of CO_2. It is possible that some CO_2 is required for the synthesis of arginine, but it may be that CO_2 is essential for other purposes. In that case it should be possible to demonstrate an effect of CO_2 in "arginine medium" although the action of arginine dihydrolase of the inoculum will result in the production of CO_2 from arginine in the medium.

The experiment described above with CO_2-free "ornithine medium" was repeated with "arginine medium." In every case the flask gassed with CO_2 showed growth before that gassed with CO_2-free N_2, although the delay between the appearance of growth in the first and in the second varied in different tests from 6 hours to 3 days. The delay was more consistent when "arginine-peptide" was used as the source of arginine as, presumably, CO_2 production is slower in this case. In one case only did the delay last as long as 4 days; the flask showing no growth was then gassed with CO_2 and growth took place within 24 hr.

The results indicate that Group D streptococci resemble other organisms in requiring CO_2 for growth. The fermentation of glucose by these organisms does not give rise to CO_2, but CO_2 is formed in normal media by the action of arginine dihydrolase on arginine.

The Formation and Activity of Arginine Dihydrolase during Growth.

Growth in "Arginine Medium."

The organism ST can grow in basal medium if either arginine or ornithine is added, but growth is more rapid and the final crop heavier with arginine than with ornithine.

When growth takes place in arginine the action of arginine dihydrolase converts the arginine to ornithine and ammonium carbonate. Fig. 5 shows the course of the growth and arginine disappearance in three cultures grown in basal medium with initial arginine concentrations equivalent to 90, 65 and 15 μl. CO_2/ml. respectively. The concentration of arginine falls very rapidly during the early stages of growth, and ornithine estimations show that quantitative conversion to ornithine takes place. The rate of growth is dependent on the arginine concentration and decreases markedly when the arginine of the medium is exhausted. The slow rate of growth which occurs after the exhaustion of the arginine presumably takes place at the expense of ornithine and CO_2, and the concentration of the latter will become progressively smaller as the pH of the medium falls due to fermentation. It has been shown above that the heaviest crops are obtained when the initial arginine concentration is such that the limit of acid tolerance is reached before all the arginine is decomposed.

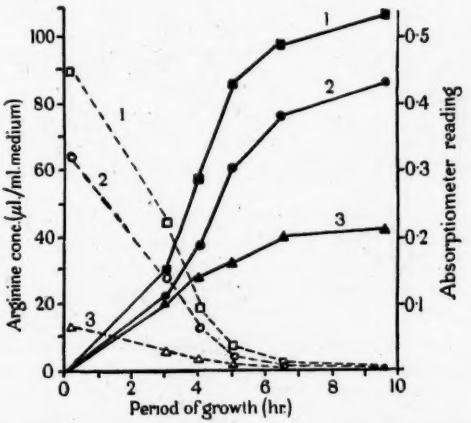

FIG. 5.—Effect of arginine concentration in medium on growth of organism ST. - - - - - - fall in arginine conc. during growth; ——— growth of organism. Initial conc. arginine (μl/ml.); Curve (1) = 90; (2) = 65; (3) = 15.

The rapid disappearance of arginine during the early phases of growth is surprising, so it was decided to investigate the variation of arginine dihydrolase activity with the age of the culture. Two litres of medium with an initial arginine concentration of 1 mg./ml. (initial pH 7·4) were inoculated and incubated as usual and samples removed with aseptic precautions at intervals throughout the growth period. Washed suspensions were prepared from each sample and the activity of the organisms towards arginine and glucose determined. Fig. 6 gives the results obtained in one such experiment, and shows that the arginine dihydrolase and fermentation enzymes are most active in organisms harvested early in the growth period, and that the activity or formation of these enzymes decreases steadily throughout the period of growth.

This type of variation of enzyme activity with age of culture is different from that recorded for many other bacterial enzymes by Gale (1943), who found that organisms harvested early in the growth period are almost inactive, and that activity develops during growth and reaches a maximum at about the time when active cell division ceases. The tyrosine decarboxylase of organism ST grown in a medium containing

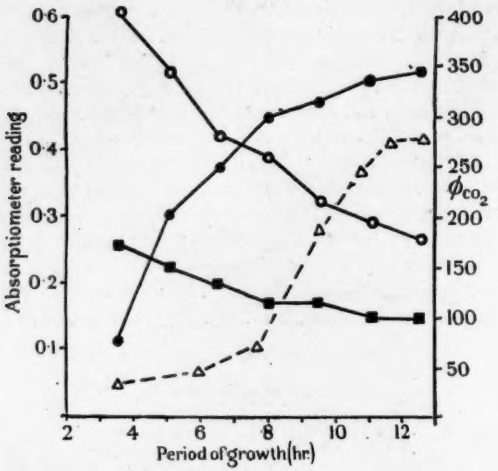

FIG. 6.—Variation of enzyme activities with age of culture (organism ST). ——○—— Q_{CO_2} acid (fermentation); ——■—— Q_{CO_2} (arginine dihydrolase); - - Δ - - Q_{CO_2} tyrosine decarboxylase); ——●—— growth of organism.

co-decarboxylase factors displays this latter type of variation (see the dotted curve in Fig. 6 and Gale, 1940). The shape of the two types of curve suggests that enzymes of the nature of arginine dihydrolase and of the fermentation system are more important to the organism during active division and the anabolic phase of metabolism than enzymes of the nature of tyrosine decarboxylase, etc.

TABLE II.—*Variation of Arginine Dihydrolase Activity with Age of Culture in Ornithine and Arginine Media (Organism ST).*

Age of culture (hr.).	"Ornithine medium"		"Arginine medium"	
	Growth (mg./ml.)	Q_{CO_2} (arginine dihydrolase).	Growth (mg/.ml.).	Q_{CO_2} (arginine dihydrolase).
3	0·085	53	0·06	205
5	0·135	35	0·15	180
8	0·170	26	0·23	135
11	0·180	8	0·26	120
24	0·188	1	0·29	85

Growth in "Ornithine Medium."

Table II shows the variation with age of culture of the arginine dihydrolase activity of organism ST grown in "ornithine medium"; corresponding values for growth in "arginine medium" are given for comparison. The enzyme shows the same type of variation with age of culture as that found during growth in "arginine medium," but the activity is considerably less at all stages and falls off more rapidly as the culture grows.

Attempts at Reversal of Arginine Dihydrolase.

If arginine is synthesized by the organism from ornithine, this process might take place by the reversal of arginine dihydrolase. Using thick suspensions of organism ST attempts have been made to demonstrate such a reversal by following the gas change in manometers containing M/8 ornithine and M/8 $(NH_4)_2SO_4$ in $NaHCO_3$ buffer systems using gas mixtures of CO_2/N_2 containing 5 and 10 per cent CO_2; no evidence for a reversal involving CO_2 uptake could be obtained. By following the forward reaction (the breakdown of arginine to ornithine and ammonium carbonate) it was not possible to show any alteration in the rate or extent of the breakdown by the addition of ornithine in concentrations up to ten times that of the initial arginine concentration. If the reverse reaction does occur, it is on too small a scale to be demonstrable by ordinary manometric methods. Estimations of the ornithine content of "ornithine medium" before and after growth of organism ST show no decrease within experimental error as a result of growth.

SUMMARY.

(1) Streptococci (Lancefield Group D) are nutritionally exacting towards arginine or ornithine.

(2) The amount of arginine required for optimal growth is dependent upon the arginine dihydrolase activity of the organism; organisms with high arginine dihydrolase activities require concentrations of the order of 0·1 per cent arginine in the medium for optimum growth.

(3) Arginine is utilized more efficiently if it is supplied in the medium in the form of hydrolysable peptide.

(4) In the absence of arginine, growth can take place in the presence of ornithine; the rate of growth and the total crop then depend upon the concentration of CO_2 in the medium.

(5) Group D streptococci are nutritionally exacting towards CO_2; when arginine is present in the medium, CO_2 is formed by the action of arginine dihydrolase.

(6) Arginine dihydrolase is most active in cells harvested early in the growth phase and the activity per mg. dry weight of organism falls off steadily during growth. The fermentation enzyme system displays a similar type of variation with the age of the culture.

(7) It has not been possible to demonstrate a reversibility of arginine dihydrolase.

The author wishes to thank Professor A. C. Chibnall and Dr. M. Stephenson for helpful criticism. He is a member of the Scientific Staff of the Medical Research Council.

REFERENCES.

Bellamy, W. D., and Gunsalus, I. C.—(1944) *J. Bact.*, **48**, 191.

Gale, E. F.—(1940) *Biochem. J.*, **34**, 846.—(1943) *Bact. Rev.*, **7**, 139.—(1945) *Biochem. J.*, **39**, 46.

Idem and Epps, H. M. R.—(1944) *Ibid.*, **38**, 232.

Gladstone, G. P., Fildes, P., and Richardson, G. M.—(1935) *Brit. J. exp. Path.*, **16**, 335.

Hills, G. M.—(1940) *Biochem. J.*, **34**, 1057.

McIlwain, H.—(1940) *Brit. J. exp. Path.*, **21**, 25.

Niven, C. F., and Sherman, J. M.—(1944) *J. Bact.*, **47**, 335.

Schuman, R. L., and Farrell, M. A.—(1941) *J. infect. Dis.*, **69**, 81.

Taylor, A. E.—(1908) *J. biol. Chem.*, **5**, 389.

Valley, G., and Rettger, L. F.—(1926) *J. Bact.*, **11**, 78.—(1927) *Ibid.*, **14**, 101.

THE EFFECT OF SULPHANILAMIDE ON THE ARGININE AND CARBON DIOXIDE REQUIREMENTS OF STREPTOCOCCI (LANCEFIELD GROUP D).

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DURING studies of the arginine requirements of Group D streptococci (Gale, 1945) it was noticed that these organisms, although sulphonamide resistant, are under certain circumstances sensitive to sulphanilamide. Further, the inhibition of growth is not antagonized in the usual manner by *p*-aminobenzoic acid. This communication deals with the investigation of these effects, and shows that sulphanilamide, but not substituted sulphonamides, inhibits the metabolism of CO_2 by Group D streptococci, and that this effect is apparently supplementary to the competitive inhibition of *p*-aminobenzoic acid metabolism first demonstrated by Woods (1940).

EXPERIMENTAL.

Organism and Methods.

Organism.—The results quoted in this paper were obtained with organism ST, a non-haemolytic Group D streptococcus (N.C.T.C. No. 6782) described in the previous paper (Gale, 1945).

Media.—The basal medium described by Gale (1945) was used throughout the work; either 0·1 per cent arginine or 0·04 per cent ornithine was added as required, and such media are referred to in the text as "arginine medium" or "ornithine medium." In all cases media were inoculated with 0·5 per cent of their volume of a 16 hr. culture of organism ST in casein digest-glucose-marmite broth; the inoculum was *ca.* 10^6 viable cells/ml. of final test medium. Incubation was at 37° C. When experiments required that samples should be taken at intervals throughout growth, the media were put up in 100 ml. flasks fitted with gassing and sampling tubes: samples were withdrawn by closing the gas outlet, and opening the sampling siphon-tube when the increased pressure within the flask forced medium through the siphon under aseptic conditions.

Estimation of growth.—Growth was estimated as described in the previous paper (Gale, 1945).

Estimation of arginine.—Arginine was estimated manometrically by the use of an arginine decarboxylase preparation (Gale, 1945).

Growth at constant pH.—Since the basal medium contains glucose, growth is normally accompanied by a fall in the pH due to fermentation acids. In some cases it was desirable to check results by growth at a constant pH; for this purpose the medium was made up in the apparatus described by Gale and van Heyningen (1942) which

incorporates a hydrogen electrode in the medium ; the pH is estimated by a valve-potentiometer, and the pH maintained at a constant value throughout growth by the controlled addition of sterile alkali or acid.

The Effect of Sulphanilamide on Arginine Requirement.

Fig. 1 shows that the total crop of organism ST growing in " arginine medium " is decreased by the addition of sulphanilamide. A concentration of 1 g./l. (0·0058M) results in a decrease of approx. 60 per cent in the crop of organism. The addition of *p*-aminobenzoic acid, 1 mol. per 100 mol. sulphanilamide, neutralizes the inhibitory effect.

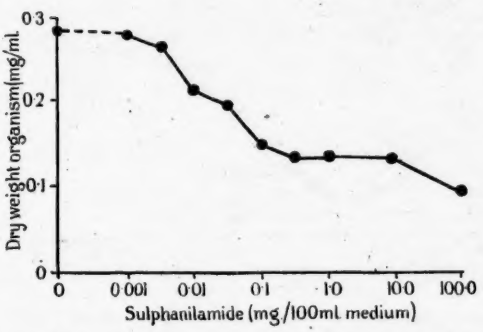

FIG. 1.—Effect of sulphanilamide concentration on the total crop of organisms produced in " arginine medium." Growth estimated after 16 hr. incubation.

Fig. 2 shows the growth of the organism in " arginine medium " with and without 0·1 per cent sulphanilamide. The action of sulphanilamide is to reduce the rate of growth in the early stages. If the arginine content of the medium is estimated at intervals during growth it is found that growth ceases very soon after the arginine is exhausted ; in the presence of sulphanilamide the rate of growth is slower, and since, as shown below, the arginine dihydrolase activity is unaffected, the arginine suffices to produce a smaller total crop of organism than that which is obtained in the absence of the inhibitor. In the uninhibited growth, once the arginine is exhausted, a slower growth on ornithine and CO_2 continues until the pH of the medium reaches the limiting acid tolerance of the organism (Gale, 1945). The cessation of growth in the sulphanilamide culture is not due to the limiting acidity being reached, since pH measurements show that growth ceases at pH 4·5 in the absence of sulphanilamide, but at pH 6·5 in its presence. That the cessation is due to exhaustion of arginine is proved (curve 3, Fig. 2) by the addition of arginine to the medium containing sulphanilamide just as growth has ceased ; growth then starts again and continues as long as any arginine remains unchanged in the medium or until the limiting pH is reached, whichever takes place first. In the presence of sulphanilamide, it appears that the growth of the organism is exacting towards arginine.

The decreased crop in the presence of sulphanilamide is thus an indirect consequence of the slower rate of growth. This slower rate of growth might be due to interference with the supply of energy or of some essential metabolite. Table I shows that the presence of sulphanilamide does not inhibit either the formation or the activity of arginine dihydrolase or of the enzymes involved in the fermentation of glucose to lactic

acid. The results were obtained by the preparation of two identical batches of "arginine medium," to one of which 0·1 per cent sulphanilamide was added. The media were inoculated and samples withdrawn at intervals during the growth period.

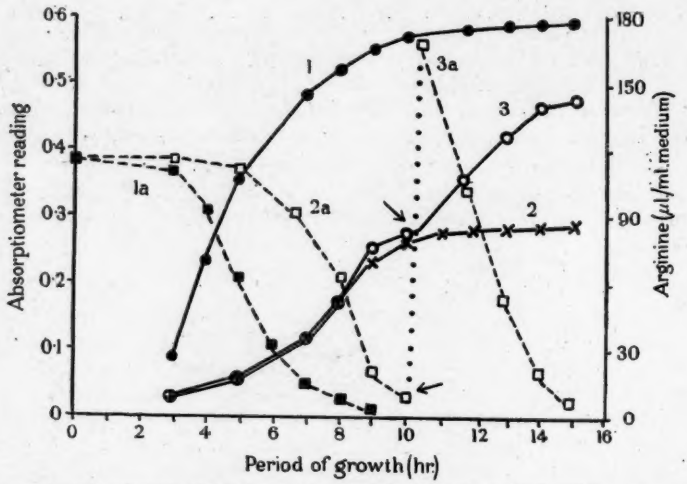

FIG. 2.—Effect of sulphanilamide (0·1 per cent) on growth and arginine utilization. ———, growth curves; - - - - - - - -, arginine content of medium during growth; (1) and (1*a*), "arginine medium"; (2) and (2*a*), arginine + sulphanilamide medium; (3) and (3*a*), arginine + sulphanilamide medium; 170μl. extra arginine added after 10 hr. (as indicated by arrows).

The organisms were centrifuged down, washed once, resuspended, and their activities towards arginine and glucose determined as described previously (Gale, 1945). As the rates of growth in the two media are so different, results for QCO_2 (arginine) and QCO_2

TABLE I.—*Absence of Effect of Sulphanilamide on Formation and Activity of Arginine Dihydrolase and Fermentation Enzyme Systems.*

Medium = basal + 0·1 per cent arginine.

Sulphanilamide in medium.	+	—	+	—
Absorptiometer readings:	Q_{CO_2} (arginine dihydrolase).		$Q_{CO_2}^{acid}$ (fermentation).	
0·07	280	—	560	—
0·107	—	205	—	485
0·115	238	—	436	—
0·200	199	—	394	—
0·220	205	—	378	—
0·301	—	180	—	405
0·340	137	—	314	—
	131*		320*	
0·372	—	162	—	314
		156*		312*

* Value determined in presence of 0·1 per cent sulphanilamide.

(glucose) are correlated with the degree of growth (absorptiometer readings) rather than with time of growth. On this basis it can be seen that there is no significant difference in the rate of formation or rate of variation with age of culture of either enzyme in the two media. In each case the activity of the last suspension to be prepared was redetermined in the presence of 0·1 per cent sulphanilamide and no significant inhibition was obtained. Consequently the action of sulphanilamide on the rate of growth of the streptococci does not involve inhibition of the formation or activity of these enzyme systems.

Free or combined nature of arginine.—It has been shown in the previous paper (Gale, 1945) that the organism utilizes arginine more efficiently if this is supplied in the form of a hydrolysable peptide than when supplied as the free amino acid. Table II shows that sulphanilamide is less inhibitory when the source of arginine in the medium is in the form of an arginine-peptide than when it is present as the free amino acid. The quantities of arginine used are those previously demonstrated to produce the same crop of organism ST under normal growth conditions.

TABLE II.—*Variation of Sulphanilamide Inhibition with Source of Arginine in the Medium.*

Nature of arginine source.	Optimal growth mg./ml. in medium.		Per cent decrease in growth in presence of sulphanilamide.
	Without sulphanilamide.	With sulphanilamide.*	
0·1 per cent free arginine	0·400	0·192	52
0·01 per cent arginine-peptide (Salmin preparation)	0·360	0·299	17

* Final concentration in medium, 0·1 per cent.

The Inhibition of Growth in Arginine Media by Sulphanilamide and its Antagonism by p-*Aminobenzoic Acid.*

The effect of *p*-aminobenzoic acid on the inhibition by sulphanilamide of the growth of the test organism on " arginine medium " is shown in Fig. 3.

The presence of sulphanilamide results in a decreased rate of growth (compared with the control) in the early stages of culture, followed by an increasing rate of growth which persists until exhaustion of the arginine in the medium and consequent cessation of growth as shown above. The presence of *p*-aminobenzoic acid in addition to sulphanilamide results in the ultimate production of a normal final crop, but the antagonistic action of the *p*-aminobenzoic acid is not displayed until late in the growth period when growth has almost ceased in the sulphanilamide culture. In the experiment illustrated the *p*-aminobenzoic acid has no observable effect for the first 8½ hr. of culture. In the many studies that have been made during recent years of the growth of various organisms in the presence of sulphanilamide and *p*-aminobenzoic acid, there is no record of a similar lag period being observed in the antagonistic action of *p*-aminobenzoic acid. In the example quoted, growth took place with air over the media in the flasks, and the initial pH of the medium was 7·4.

The organism ST has three known metabolic activities: it ferments glucose to lactic acid and no other product, it possesses arginine dihydrolase which decomposes arginine to ornithine and ammonium carbonate, and it also possesses tyrosine decarboxylase (Gale, 1940). When the organism is grown, as described here, in a medium containing little tyrosine, the tyrosine decarboxylase activity is negligible. The appearance of the *p*-aminobenzoic acid growth curve suggests that some physical or

chemical change occurs in the medium at 8½ hr., and that until that change has occurred, *p*-aminobenzoic acid is not effective as sulphanilamide antagonist. A number of possibilities are examined below.

(1) *Concentration of* p-*aminobenzoic acid.*—The experiment described was repeated with concentrations of *p*-aminobenzoic acid corresponding to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the concentration of sulphanilamide present. In all cases controls containing "arginine medium" plus corresponding concentrations of *p*-aminobenzoic acid in the absence of sulphanilamide were carried out, but in no case was any significant effect obtained on the growth by such addition. In general such control curves are omitted from figures for the sake of clarity. Variation of the *p*-aminobenzoic acid concentration does not alter the lag-period before sulphanilamide antagonism appears, and

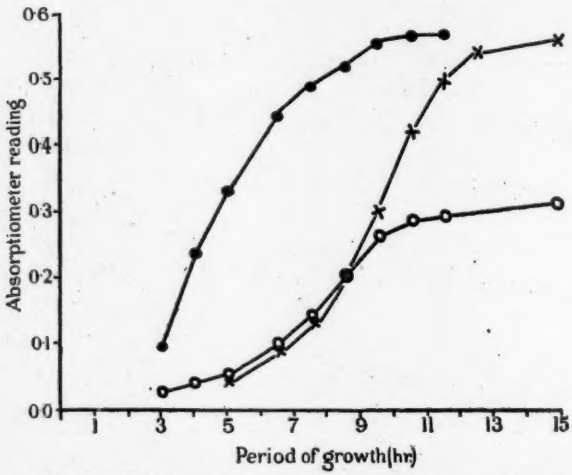

FIG. 3.—Effect of *p*-aminobenzoic acid on the inhibition by sulphanilamide of growth in "arginine medium." ——●——, "arginine medium" (control); ——○——, "arginine medium" + 0·1 per cent sulphanilamide; ——×——, "arginine medium" + 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid.

the curves in all cases are essentially similar to those shown in Fig. 3, except that the lowest concentration of *p*-aminobenzoic acid used does not fully antagonize the inhibition of total growth due to sulphanilamide.

(2) *Concentration of ornithine.*—The action of arginine dihydrolase on arginine produces ornithine; it is possible that a threshold ornithine concentration must be reached before *p*-aminobenzoic acid is effective. The experiments were repeated with media to which 0·04 and 0·08 per cent ornithine were added, in addition to arginine, but there was no alteration in the length of the lag period as a result.

(3) *Variation of pH of medium.*—In the experiments quoted above the pH of the medium prior to inoculation was 7·4; during growth glucose is fermented to lactic acid and the pH falls to between 4·5 and 5·0 by the twelfth hour. The antagonistic effect of *p*-aminobenzoic acid begins when the pH in the medium has fallen to 6·8. A series of experiments was set up in which the initial pH of the medium was adjusted to 7·6, 7·3, 7·1, 6·8 and 6·35 respectively.

TABLE III.—*Anti-sulphanilamide Activity of* p-*Aminobenzoic Acid with Varying pH of Medium.*

All experiments in " arginine medium."

Growth measurements are recorded as absorptiometer readings; column a = medium + 0·1 per cent sulphanilamide; column *b* = medium + 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid. Italicized figures in column *b* indicate that *p*-aminobenzoic acid is exerting anti-sulphanilamide effect.

pH.	Experiment. 1		2		3		4		5	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
7·6	—	—	..	..	..	..	..	..	..	..
7·5	0·06	0·04	..	..	..	..	..	..	..	..
7·4	0·06	0·04	..	..	..	..	..	..	..	..
7·3	0·16	0·15	—	—	..	..	..	..	..	..
7·2	0·23	0·22	0·05	0·05	..	..	..	..	..	..
7·1	0·30	0·29	0·05	0·05	—	—	..	..	..	..
7·0	0·32	0·30	0·10	0·10	0·04	0·04	—	—	..	..
6·9	0·33	0·31	0·16	0·16	0·09	0·10	0·05	0·04	..	..
6·8	0·33	*0·37*	0·25	*0·29*	0·20	0·21	0·05	0·04	..	..
6·7	0·33	*0·43*	0·27	*0·38*	0·32	*0·40*	0·15	0·13	..	..
6·6	..	..	0·31	*0·45*	0·32	*0·42*	0·26	*0·29*	..	..
6·5	..	..	..	..	0·32	*0·44*	0·30	*0·40*	..	..
6·4	..	..	..	..	0·32	*0·45*	0·30	*0·46*	—	—
6·3	..	..	..	..	0·32	*0·47*	0·30	*0·48*	0·05	*0·08*
6·2	..	..	..	..	0·32	*0·48*	0·30	*0·50*	0·14	*0·19*
6·1	..	..	..	..	0·32	*0·50*	..	..	0·32	*0·38*
6·0	..	..	..	..	..	..	..	..	0·35	*0·45*
5·9	..	..	..	..	..	..	..	..	0·37	*0·51*
5·8	..	..	..	..	..	..	..	..	0·39	*0·56*

Figs. 4 and 4*a* show the results obtained in the two limiting cases, and Table III shows the pH of the medium in each case when the anti-sulphanilamide action of *p*-aminobenzoic acid first begins to appear. In every case *p*-aminobenzoic acid is ineffective until the medium pH has fallen to 6·9–6·6. When the initial pH was 6·35 *p*-aminobenzoic acid had some antisulphanilamide activity from the time of the earliest readings recorded (3 hr.). When the initial pH was 7·6, *p*-aminobenzoic acid had no anti-sulphanilamide effect until growth had almost ceased at the twelfth hour, when the pH dropped to 6·9 and the growth in the presence of *p*-aminobenzoic acid increased over that in the presence of sulphanilamide alone. The length of the lag period is thus determined by the initial pH, and the antisulphanilamide effect does not take place until the pH has fallen below 6·9.

(4) *Concentration of arginine.*—It has been shown above that the organism ST is exacting towards arginine in the presence of sulphanilamide under the conditions described. The results obtained by variation of the initial pH suggest that should the initial pH be such that the pH of the medium has not fallen below 6·9 by the time the arginine is exhausted, then *p*-aminobenzoic acid would have no anti-sulphanilamide effect. This is confirmed by the results given in Fig. 5 showing the total crop obtained in basal medium plus various concentrations of arginine, (*a*) alone, (*b*) with 0·1 per cent sulphanilamide, and (*c*) with 0·1 per cent sulphanilamide and 0·001 per cent *p*-amino-

benzoic acid. In the presense of high concentrations of arginine and at an initial pH of 7·5, *p*-aminobenzoic acid is completely antagonistic to sulphanilamide. As the

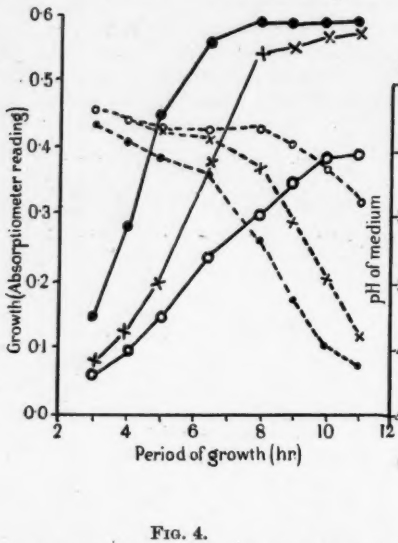

FIG. 4.

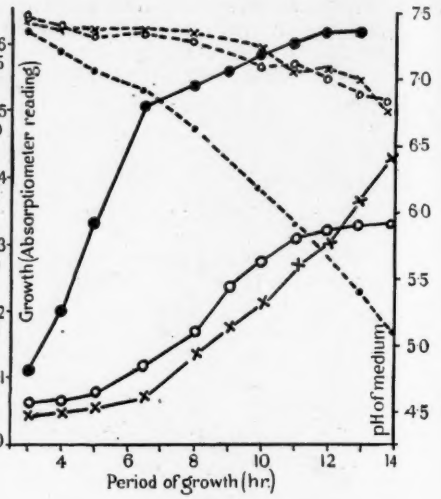

FIG. 4*a*.

FIGS. 4 and 4*a*.—Correlation of pH with the effect of *p*-aminobenzoic acid on growth inhibition by sulphanilamide in "arginine medium." ———, growth; - - - - - - - -, pH of medium; ——●- - - -, normal; ——○- - - -, 0·1 per cent sulphanilamide; ——×- - - -, 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid. Fig. 4, initial pH = 6·35. Fig. 4*a*, initial pH = 7·6.

arginine concentration is decreased, *p*-aminobenzoic acid becomes less and less effective until, for concentrations of less than 0·025 per cent arginine, it has no anti-sulphanilamide action. In these last cases the medium pH remains above 7·0 until after exhaustion of the arginine.

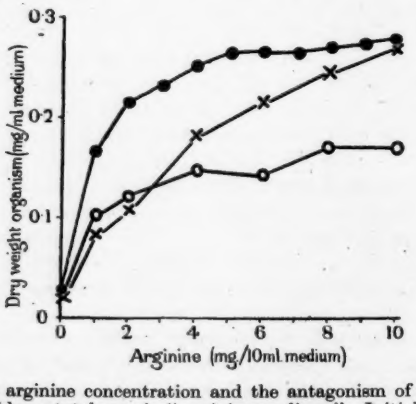

FIG. 5.—Relation between arginine concentration and the antagonism of *p*-aminobenzoic acid to the effect of sulphanilamide on total crop in "arginine medium." Initial pH of all media = 7·5; control = "arginine medium" alone. ——●——, control; ——○——, 0·1 per cent sulphanilamide; ——×——, 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid.

The Inhibition of Growth in " Ornithine Medium " by Sulphanilamide and the Action of p-*Aminobenzoic Acid.*

The experiments described in the previous section were next repeated under similar conditions in basal medium plus ornithine instead of arginine. Figs. 6 and 6*a* show the results obtained for two experiments in which the initial pH was 6·4 and 7·7 respectively. Sulphanilamide inhibits the rate of growth, but this inhibition is unaffected by the presence of *p*-aminobenzoic acid at any pH. In some cases a slightly accelerated growth is promoted in the presence of *p*-aminobenzoic acid which may, in the course of 24–30 hr., result in a total crop approaching that obtained in the absence of sulphanilamide, but in no case is there ever a sudden acceleration of growth as obtained in the " arginine medium " when the pH falls below 6·9. In these experiments growth continues very slowly for many hours until eventually the pH of the medium falls to *ca.* 4·4.

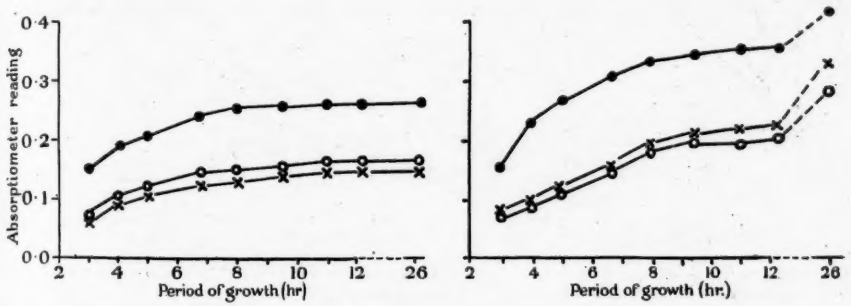

FIG. 6. FIG. 6*a*.

FIGS. 6 and 6*a*.—Effect of sulphanilamide and *p*-aminobenzoic acid on growth in " ornithine medium." —●—, normal ; —○—, 0·1 per cent sulphanilamide ; —×—, 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid. Fig. 6, initial pH = 6·4. Fig. 6*a*, initial pH = 7·7.

The Effect of Carbon Dioxide on Growth Inhibition by Sulphanilamide and its Antagonism by p-*Aminobenzoic Acid.*

p-Aminobenzoic acid has a dramatic anti-sulphanilamide action in the " arginine medium " when the pH has fallen below 6·9, but little or no action in " ornithine medium." The main difference between growth and metabolism in these media is that whereas in " ornithine medium " the growth of the organism is exacting towards CO_2, in " arginine medium " the action of arginine dihydrolase provides a steady supply of CO_2 from the decomposition of arginine. It was decided therefore to repeat the experiments in " ornithine medium " in the presence of carbon dioxide.

Media were made up in duplicate in flasks fitted with gassing heads. One series was gassed with N_2 freed from CO_2 by passage through washing bottles containing NaOH and through a soda-lime tower ; the other series was gassed with 5 per cent CO_2 in N_2. The passage of the CO_2 through the medium lowers the pH about 0·3 unit, and consequently the media involved were adjusted before inoculation to 7·7 as compared with 7·4 in the N_2 series. Fig. 7 shows that, in the absence of CO_2, 0·1 per cent sulphanilamide stops all growth of organism, and that *p*-aminobenzoic acid has no anti-sulphanilamide action. In the presence of 5 per cent CO_2 the inhibitory action of sulphanilamide is greatly decreased, and is completely antagonized by *p*-aminobenzoic

acid. There is some difference in the growth of the two normal cultures, but this is not very great.

The experiments were next repeated with arginine in the medium instead of ornithine. In this case, CO_2 is produced in the medium by the action of arginine dihydrolase, and no significant difference is found between normal cultures gassed with either N_2 or CO_2/N_2 mixtures. However, in the presence of 0·1 per cent sulphanilamide, no growth takes place when the medium is gassed with N_2, but only 20–30 per cent inhibition of final crop is obtained when the medium is gassed with 5 per cent CO_2 in N_2. Further, the inhibition obtained in the absence of CO_2 is not antagonized by *p*-aminobenzoic acid while full antagonism is given in the presence of CO_2.

The effect of the concentration of CO_2 in the gas phase on the inhibition of growth in "arginine medium" by sulphanilamide (0·1 per cent) was next studied. Gas mixtures containing 0, 5 and 10 per cent CO_2 in N_2 were used. Increasing the CO_2 concentration from 5 to 10 per cent results in a further decrease in the sulphanilamide effect. The residual inhibition can again be antagonized by *p*-aminobenzoic acid.

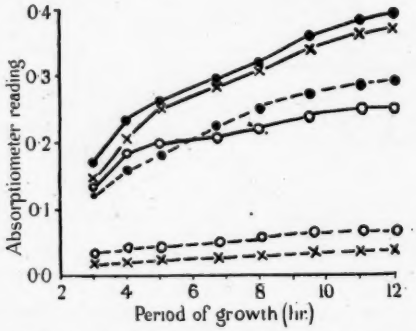

FIG. 7.—Effect of CO_2 on the sulphanilamide–*p*-aminobenzoic acid relationship ("ornithine medium"). Gas phase: - - - - - -, CO_2-free N_2; ——, 5 per cent. CO_2 in N_2. ——●- - - -, normal; ——○- - -, 0·1 per cent sulphanilamide; ——×- - - -, 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid.

In the early experiments in which the atmosphere over the cultures was air, it has been shown that *p*-aminobenzoic acid has no anti-sulphanilamide effect until the pH has fallen below 6·9. In these experiments the possibility arises that the gassing with CO_2 does, despite the preliminary adjustment, activate the *p*-aminobenzoic acid by lowering the medium pH below the critical value, although this will not explain the results obtained in the absence of CO_2. Consequently the experiments were repeated at constant pH. Six sets of apparatus for growth at constant pH were set up with the three media (normal, sulphanilamide, sulphanilamide + *p*-aminobenzoic acid) in duplicate. CO_2-free N_2 was passed through one series, and 10 per cent CO_2 in N_2 through the other; the flasks were immersed in a thermostat at 37° C., and the pH of all flasks stabilized by the addition of sterile alkali before inoculation. Two series were performed; in one the pH was maintained at 7·15 and in the other at 7·8. "Arginine-medium" was used in all cases. Fig. 8 shows that essentially similar results are obtained when the pH is stabilized as when it is not. The figures proved that (1) the inhibition of growth by sulphanilamide is antagonized by CO_2; (2) this antagonism is not complete and depends upon the CO_2 concentration and the pH, a given CO_2 concentration being less effective as the pH rises; (3) *p*-aminobenzoic

acid is not effective in the absence of CO_2; and (4) in the presence of CO_2 the growth inhibition remaining is completely antagonized by *p*-aminobenzoic acid, this effect being independent of pH values below 7·8 (as far as tested).

In the light of these findings, it is possible to interpret the curves (Figs. 3 and 4) obtained for growth and inhibition of growth in air. In "arginine medium" CO_2 is supplied to the organism by the decomposition of arginine by arginine dihydrolase, but from no other source (no CO_2 is produced in the fermentation of glucose), and consequently the CO_2 concentration in the medium is low, particularly in the early stages of growth. The initiation of anti-sulphanilamide activity by *p*-aminobenzoic acid takes place when the pH of the medium falls to 6·9–6·7, suggesting a change in ionization of some substance involved in the reaction: the pK_a for *p*-aminobenzoic acid is

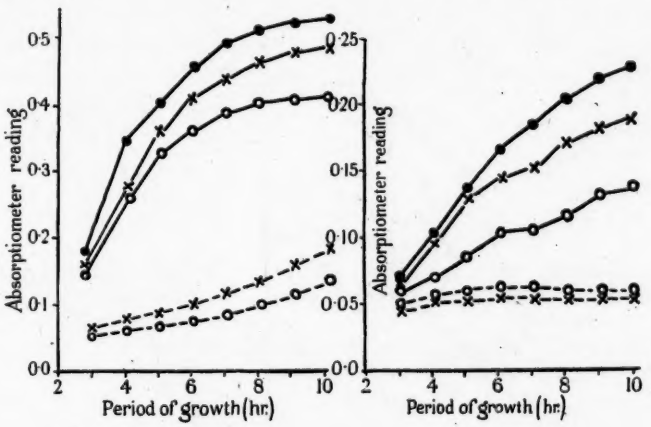

FIG. 8. FIG. 8*a*.

FIG. 8.—Action of sulphanilamide and *p*-aminobenzoic acid on growth in "arginine medium" at constant pH with and without CO_2. Gas phase: ———, 10 per cent CO_2 in N_2; - - - - - -, CO_2-free N_2. ——●- - -·, normal; ——○- - -·, 0·1 per cent sulphanilamide; ——×- - -·, 0·1 per cent sulphanilamide + 0·001 per cent *p*-aminobenzoic acid. Fig. 8, pH stabilized at 7·15. Fig. 8*a*, pH stablized at 7·8. Normal (CO_2-free) curves omitted as not significantly different from normal (CO_2).

4·68, so that no rapid change in the acid ionization of this substance is occurring at the critical pH, but the pK for $H_2CO_3 = HCO_3' + H^+$ is 6·45, so that the ionization of carbonic acid is being suppressed in favour of free CO_2 at this stage of growth. It would seem that *p*-aminobenzoic acid exerts an anti-sulphanilamide action in the presence of a critical concentration of free CO_2 which, in the aerobic culture, is only reached when the pH approaches the pK of H_2CO_3; it can be seen from Fig. 3 that the sulphanilamide inhibition also decreases at this time. When the cultures are bubbled with CO_2 this critical concentration is reached at higher pH values within the range tested.

The question arises whether *p*-aminobenzoic acid is involved in CO_2 metabolism or whether we are dealing here with two different effects, one concerned with *p*-aminobenzoic acid and its competitive inhibition by sulphonamides, and one concerned with the essential CO_2 metabolism of the organism. Mann and Keilin (1940) have shown that carbonic anhydrase is inhibited by sulphanilamide, but that this inhibition is removed if the sulphanilamide molecule is substituted in the $-SO_2NH_2$ group. Sub-

stitution in the *para*-NH_2 group removes the bacteriostatic action of sulphanilamide, but such compounds are still active as inhibitors of carbonic anhydrase.

The Effect of Carbon Dioxide on Growth Inhibition by Substituted Sulphanilamides.

Acetyl- and benzyl-sulphanilamide have no inhibitory effect upon the growth of organism ST in "arginine medium." Sulphathiazole, sulphapyridine and sulphacetamide are all inhibitory, and their action is antagonized by *p*-aminobenzoic acid in suitable concentrations. The rate of growth and total crop in "arginine medium" in the presence of each of these sulphonamides with and without *p*-aminobenzoic acid in the presence and absence of CO_2 has been investigated in a manner similar to that

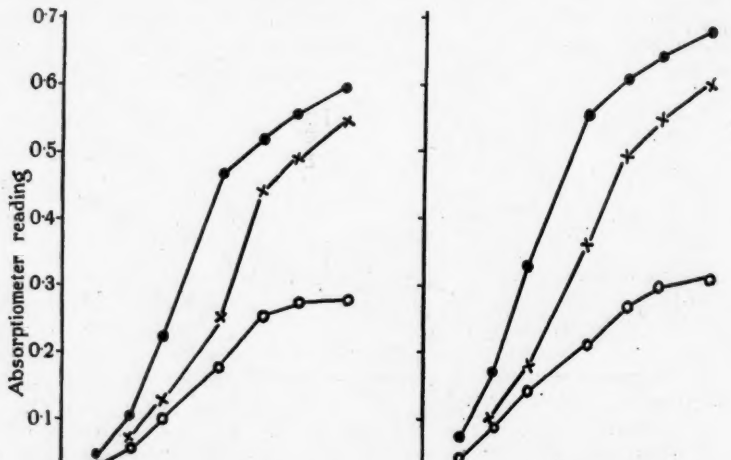

FIG. 9. FIG. 9a.

FIG. 9.—Effect of CO_2 on the action of sulphathiazole on growth in "arginine medium" in the presence and absence of *p*-aminobenzoic acid. ——●——, normal; ——○——, 0·1 per cent sulphathiazole; ——×——, 0·1 per cent sulphathiazole + 0·1 per cent *p*-aminobenzoic acid. Fig. 9, gas phase: CO_2-free N_2. Fig. 9a, gas phase: 10% CO_2 in N_2.

described above for sulphanilamide. Fig. 9 shows the results obtained with 0·1 per cent sulphathiazole and 0·1 per cent *p*-aminobenzoic acid. The removal or addition of CO_2 has no significant effect on the inhibition of growth or its antagonism by *p*-aminobenzoic acid. Table IV summarizes the results obtained with the substituted sulphanilamide derivatives; only with sulphanilamide itself has CO_2 any significant effect on the inhibition of growth. In the presence of CO_2 the order of effectiveness of the four sulphonamides against organism ST is the same as that determined by other workers against organisms in general (*cf.* Bell and Roblin, 1942), but when CO_2 is removed from the growth medium, sulphanilamide becomes the most active inhibitor of the four while the activities of the three substituted compounds remain unaltered. No effect of CO_2 could be shown on the inhibition produced by Marfanil or the related drugs V187 and V335 (Evans, Fuller and Walker, 1944).

TABLE IV.—*Effect of CO_2 on Inhibition of Growth by Substituted Sulphanilamide Derivatives.*

The sulphonamides were used at a final conc. of 0·1 per cent in "arginine medium." The results are expressed as per cent inhibition of total crop of organisms compared with the control without sulphonamide.

Derivative.	pK_a.*	In presence of 10 per cent CO_2. Per cent inhibition. (*a*)	Antagonism by *p*-aminobenzoic acid.	In absence of CO_2. Per cent inhibition. (*b*)	Antagonism by *p*-aminobenzoic acid.	(*b*)—(*a*).
Sulphanilamide	10·43	20	+	99	—	79
Sulphacetamide	5·38	49	+	51	+	3
Sulphapyridine	8·43	36	+	41	+	5
Sulphathiazole	7·12	55	+	61	+	6
Marfanil	..	29	—	28	—	—
Benzyl-N^1-sulphanilamide	..	*nil*	..	*nil*	..	..
Acetyl-N^1-sulphanilamide	..	,,	..	,,	..	..

* *Cf.* Bell and Roblin (1942).

DISCUSSION.

The sulphonamides act bacteriostatically upon many organisms by a mechanism best explained by Woods (1940) as a competition for the essential metabolite *p*-aminobenzoic acid. The nature of the metabolism involving *p*-aminobenzoic acid has not yet been discovered. Group D streptococci are exceptional amongst the Gram-positive cocci in being resistant to sulphonamides in general, which is confirmed by the figures given in Table IV. Sulphanilamide is a special case in that the degree of inhibition it produces is profoundly influenced by the CO_2 concentration, and that this inhibition is not antagonized by *p*-aminobenzoic acid in the absence of CO_2. The fact that the substituted sulphonamides do not show this effect, and that the growth curves obtained with them are of a different shape from those obtained with sulphanilamide (*cf.* Figs. 3 and 9), suggests that the action of sulphanilamide itself on Group D streptococci is not a simple *p*-aminobenzoic acid competition. It appears that sulphanilamide has two actions on Group D streptococci, the first in competition with the essential metabolite *p*-aminobenzoic acid, and the second in competition with CO_2. Valley and Rettger (1926, 1927) showed that many organisms, including *Strept. pyogenes*, are unable to grow in the absence of CO_2, and this fact was confirmed under more exact conditions by Gladstone, Fildes and Richardson (1935), although none of these workers tested Group D streptococci. Gladstone *et al.* were able to show a complete inhibition of growth with many organisms growing in a simple medium, but were only able to show a considerably delayed growth in the absence of CO_2 with organisms which would grow only in comparatively complicated media, and in which some production of CO_2 occurred. Gale (1945) has shown that Group D streptococci will grow in "ornithine medium" only as long as CO_2 is present, and that the rate of growth is dependent upon the CO_2 concentration; on "arginine medium" CO_2 is formed by the action of arginine dihydrolase, but if this CO_2 is removed during incubation there is a significant delay in the onset of growth.

The inhibition of growth produced in "arginine medium" by sulphanilamide can be antagonized by CO_2; since the degree of antagonism is dependent upon the concentration of unionized CO_2, it is possible that sulphanilamide competitively inhibits an

enzyme system involved in the essential metabolism of CO_2. It is not possible to state definitely whether *p*-aminobenzoic acid opposes this sulphanilamide effect; *p*-aminobenzoic acid certainly has no anti-sulphanilamide action in the absence of CO_2 with this organism, but if CO_2 is the substrate of the enzyme system inhibited, then no anti-sulphanilamide effect of *p*-aminobenzoic acid could in any case be demonstrated in the absence of the substrate of the enzyme.

The work described in the early part of this paper on the effect of sulphanilamide on arginine requirement shows that the effect of CO_2 cannot be ascribed to inhibition of arginine dihydrolase. It was also shown that the growth of Group D streptococci under the conditions described was apparently exacting towards arginine when 0·1 per cent sulphanilamide was present in the medium. The probable explanation of this finding is that when growth takes place in arginine in the absence of sulphanilamide, it continues after exhaustion of the arginine by utilization of CO_2 and ornithine but, under the experimental conditions used, the supply of CO_2 is restricted by the increasing acidity of the medium; the presence of sulphanilamide still further restricts the utilization of CO_2, so that bacteriostasis results as soon as the arginine is exhausted.

For the present, it is suggested that the results obtained in these studies may be explained as follows: (1) Group D streptococci are exacting towards CO_2, which is normally produced by the action of arginine dihydrolase on arginine in the medium; (2) sulphanilamide acts as a competitive inhibitor of an enzyme system involved in the essential metabolism of CO_2; (3) this CO_2 inhibition is specific for the sulphanilamide molecule, and does not appear to be involved in the metabolism of *p*-aminobenzoic acid that is competitively inhibited by sulphonamides in general.

SUMMARY.

(1) When growth of streptococci (Lancefield Group D) takes place in the simplified medium described and in the presence of 0·1 per cent sulphanilamide, growth ceases as soon as the arginine of the medium has been exhausted. The addition of further arginine results in renewed growth.

(2) Sulphanilamide reduces the rate of growth, and this effect can be antagonized by *p*-aminobenzoic acid: under the experimental conditions described *p*-aminobenzoic acid has no anti-sulphanilamide action until the pH of the medium has fallen below 6·9–6·7. It is suggested that *p*-aminobenzoic acid has no anti-sulphanilamide action in the absence of a certain threshold concentration of CO_2.

(3) When growth takes place in a simplified medium containing ornithine instead of arginine, *p*-aminobenzoic acid has no anti-sulphanilamide action unless the medium is bubbled with CO_2.

(4) When growth occurs in either arginine or ornithine medium the inhibitory effect of sulphanilamide can be markedly increased by the removal of CO_2 from the medium: in the absence of CO_2, *p*-aminobenzoic acid has no anti-sulphanilamide effect. The inhibitory action of sulphanilamide on growth can be greatly reduced by bubbling the medium with gas mixtures containing CO_2: the degree of antagonism displayed by CO_2 to sulphanilamide depends upon (*a*) the CO_2 concentration and (*b*) the pH, a given concentration of CO_2 being less antagonistic at high pH values than low.

(5) Sulphanilamide has no inhibitory action upon the activity, formation or variation with age of culture either of arginine dihydrolase or of the enzymes involved in the *homo*lactic fermentation of glucose.

(6) The antagonism between sulphanilamide and CO_2 appears specific for sulphanilamide itself, as no such effect can be demonstrated with either N^1- or N^4-

substituted sulphanilamides, although these, if inhibitory to growth, are antagonized as usual by *p*-aminobenzoic acid.

(7) The Group D streptococci are nutritionally exacting towards CO_2, and it is suggested that sulphanilamide, though not sulphonamides in general, competitively inhibits an enzyme involved in the essential metabolism of CO_2.

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REFERENCES.

BELL, P. H., AND ROBLIN, R. O.—(1942) *J. Amer. chem. Soc.*, **64**, 2905.
EVANS, D. G., FULLER, A. T., AND WALKER, J.—(1944) *Lancet*, ii, 523.
GALE, E. F.—(1940) *Biochem. J.*, **34**, 846.—(1945) *Brit. J. exp. Path.*, **26**, 225.
Idem AND VAN HEYNINGEN, W. E.—(1942) *Biochem. J.*, **36**, 624.
GLADSTONE, G. P., FILDES, P., AND RICHARDSON, G. M.—(1935) *Brit. J. exp. Path.*, **16**, 335.
MANN, T., AND KEILIN, D.—(1940) *Nature*, **146**, 164.
VALLEY, G., AND RETTGER, L. F.—(1926) *J. Bact.*, **11**, 78.—(1927) *Ibid.*, **14**, 101.
WOODS, D. D.—(1940) *Brit. J. exp. Path.*, **31**, 74.

THE EXAMINATION OF RABBIT ANTI-A IMMUNE SERA PRODUCED BY MEANS OF AN ARTIFICIAL A-ANTIGEN.

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Specific serological reagents for the differentiation of A_1 and A_2 erythrocytes can be obtained by absorbing with A_2 cells the sera of certain persons belonging to group B. The absorption technique employed is based on the observations of von Dungern and Hirszfeld (1910) and of Landsteiner and Witt (1926) that most anti-A sera contain two kinds of anti-A agglutinins which are usually known as common *alpha* and *alpha*$_1$ agglutinin. The former agglutinin reacts with A_1 and A_2 cells, whereas *alpha*$_1$ immune-body agglutinates A_1 cells only. Landsteiner and Witt (1926) and Landsteiner and Levine (1926, 1929) observed that the sera of persons who belong to groups A_2 and A_2B sometimes contain a natural agglutinin which reacts specifically with A_1 and A_1B erythrocytes and not with those belonging to the sub-groups A_2 and A_2B. This agglutinin is known as the "irregular" iso-agglutinin *alpha*$_1$. Estimations of the frequency of occurrence of the agglutinin *alpha*$_1$ have been made by Landsteiner and Witt (1926), Wiener (1941), and by Taylor, Race, Prior and Ikin (1942), who consider that about 26 per cent of all A_2B persons and 1·6 per cent of all A_2 persons contain the irregular iso-agglutinin *alpha*$_1$ in their serum. From these figures it can be calculated that only 0·32 per cent of all persons contain the agglutinin *alpha*$_1$ in their serum. Unfortunately, however, even of this small number, many of the sera are not potent enough to be reliable test reagents. Immune-sera possessing a specific action on A_1 cells have also been obtained by the iso-immunization of A_2 individuals with A_1 blood as a result of an incorrect transfusion, or by the iso-immunization of a pregnant woman belonging to group A_2 by an A_1 foetus *in utero* (Wiener, 1941).

During the course of an investigation on the production of anti-A immune-sera in the rabbit by means of an artificial antigen prepared from A-substances of animal

and human origin (Morgan, 1943, 1944 ; Morgan and King, 1943 ; Morgan and van Heyningen, 1944 ; King and Morgan, 1944 ; Morgan and Watkins, 1944) it has been found that reagents can be prepared from these sera by absorption with A_2 cells that will differentiate A-erythrocytes belonging to the sub-groups A_1 and A_2. As absorbed sera possessing these specific properties are readily obtained and as they are more potent than natural *alpha*$_1$ preparations, these reagents may be of considerable value in serological studies that involve the differentiation of cells of the phenotypes A_1, A_2, A_1B and A_2B. A description of their preparation and properties is given.

EXPERIMENTAL.

A number of immune-sera were prepared by immunizing rabbits with an artificially prepared molecular complex of A-substance and the conjugated protein component of the O-antigen of *Bact. shigae* (Morgan, 1943 ; Morgan and Watkins, 1944). In our experience all rabbits that possess a normal anti-A agglutinin respond to immunization with the production of high titre anti-A agglutinins. The sera of animals that have received A-antigen prepared from hog gastric mucin A-substance more often than not react equally well with A_1 and A_2 erythrocytes, and in most instances cannot be absorbed with A_2 cells to yield a useful *alpha*$_1$ agglutinin. The sera of rabbits immunized with an A-antigen derived from A-substance of human origin, however, frequently show a considerable difference in the level of agglutinins reactive against A_1 and A_2 red-cells respectively, and are thus apparently more suitable for the preparation of an absorbed serum reactive against A_1 cells only. Immune-sera prepared by means of the artificial antigen of human origin usually show a higher titre against A_1 cells than do immune-sera induced by means of the hog gastric mucin-A antigen.

At first it was assumed that a large difference in the agglutination titre of the unabsorbed serum for A_1 and A_2 cells was essential for the preparation of a suitable absorbed reagent, but it has been found that sera which show a very similar end-point when titrated against A_1 and A_2 erythrocytes sometimes reveal, after absorption with A_2 cells, a 5 or 6 tube difference in the agglutination end-point with A_1 and A_2 cells.

Anti-A rabbit immune sera induced by means of an artificial antigen made from A-substance of human origin were exhausted of agglutinins reactive against A_2 cells by absorption with A_2 erythrocytes at room-temperature. Table I shows the titre of three undiluted and absorbed sera when tested with A_1 and A_2 cells. It is evident from the titration results that the sera react strongly with A_1 cells, but not at all with those belonging to the sub-group A_2.

TABLE I.—*Showing the Agglutination Titre with A_1 and A_2 Erythrocytes of Three Rabbit Immune-sera Produced against an Artificial A-antigen (human) and Absorbed with A_2 Cells.*

Serum No.	Test-cells.	Titre before absorption with A_2 cells.	Agglutination after absorption with A_2 cells. Final dilution of serum.						
			1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256
816	A_1	6,400	3	3	3	3	2	2	1
	A_2	3,200	0	0	0	0	0	0	0
817	A_1	12,800	3	3	3	3	3	2	2
	A_2	6,400	1	0	0	0	0	0	0
821	A_1	6,400	3	3	3	3	2	2	1
	A_2	3,200	0	0	0	0	0	0	0

Degrees of agglutination :
3, Clumps visible to the naked eye.
2, Large groups with many free cells.
1, A few groups of 2–3 cells.
0, No agglutination.

TABLE II.—*The Agglutination of A_1 and A_2 Erythrocytes by* (a) *Natural Human* Alpha$_1$ *Immune-body and* (b) Alpha$_1$ *Agglutinin Prepared by Absorbing Anti-A Rabbit Sera with A_2 Erythrocytes.*

Donor.	Blood group.*	Dilution of serum. Natural *alpha*$_1$ agglutinin (No. 1). 1:2	1:4	1:8	1:16	1:32	1:64	Absorbed serum 788. 1:8	1:16	1:32	1:64	1:128	1:256	Absorbed serum 816. 1:8	1:16	1:32	1:64	1:128	1:256
1	A_1	3	3	2	1	1	0	3	3	3	2	2	1	3	3	2	1	1	0
2	A_1	2	2	1	0	0	0	3	3	2	2	1	0	3	2	2	1	0	0
3	A_1	2	2	1	1	0	0	3	3	2	2	1	0	3	3	2	1	0	0
4	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	A_1	2	2	1	1	0	0	3	3	2	2	1	0	3	3	2	1	1	0
6	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	A_1	2	2	1	0	0	0	3	3	2	1	1	0	3	2	2	1	0	0

Donor.	Blood group.*	Natural *alpha*$_1$ agglutinin (No. 2). 1:2	1:4	1:8	1:16	1:32	1:64	Absorbed serum 788. 1:8	1:16	1:32	1:64	1:128	1:256	Absorbed serum 805. 1:4	1:8	1:16	1:32	1:64	1:128
9	A_1	3	3	3	2	1	0	3	3	3	2	2	1	3	3	2	2	1	0
10	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	A_1	3	3	2	2	1	0	3	3	3	3	2	1	3	3	2	2	1	1
13	A_1	3	3	2	1	1	0	3	3	3	2	1	1	3	3	2	2	1	0
14	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	A_2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	A_2	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0

* As determined by agglutination with *alpha*$_1$ agglutinin obtained from a person belonging to the sub-group A_2B.
Degrees of agglutination as in Table I.

In order to determine the suitability of these reagents for the differentiation of A_1 from A_2 cells, a series of 75 selected A-bloods which contained 35 bloods belonging to the sub-group A_2 were titrated in parallel with a natural irregular *alpha*$_1$ agglutinin obtained from a person of blood group A_2B, and with a number of specimens of anti-A rabbit sera which had been absorbed with A_2 cells as described above. The results obtained using two natural *alpha*$_1$ sera and three absorbed immune sera and sixteen bloods are shown in Table II, and demonstrate that in each instance there is complete agreement between the results obtained with the natural and immune *alpha*$_1$ agglutinin. Similar results were observed with the remaining bloods, and indicate that the results obtained using absorbed rabbit sera are entirely trustworthy, and enable erythrocytes of the phenotypes A_1 and A_2 to be readily differentiated.

The absorbed reagents can be made suitable for the differentiation of A_1B and A_2B cells if the serum obtained after treatment with A_2 cells is absorbed further with B-cells to remove the anti-B agglutinins. Table III shows the agglutination results

TABLE III.—*The Differentiation of Erythrocytes of the Genotypes A_1B and A_2B by Means of Absorbed* Alpha$_1$ *Rabbit Serum.*

Test-cells.	Agglutination with natural *alpha*$_1$.	Dilution of rabbit *alpha*$_1$ serum giving agglutination.					
		1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128.
A_1B	3	3	2	2	1	0	0
A_1B	2	2	2	1	0	0	0
A_1B	3	3	2	2	1	1	0
A_2B	0	0	0	0	0	0	0
A_2B	0	0	0	0	0	0	0
A_1B	3	3	3	2	2	1	0

Degrees of agglutination as in Table I.

obtained with an absorbed serum of this kind, and demonstrates the specificity of its action on cells of genotype A_1B and A_2B.

The pronounced thermal amplitude, that is, the variation in the end-point of agglutination with temperature, of the natural *alpha*$_1$ agglutinin in absorbed B sera and of the irregular *alpha*$_1$ agglutinin occurring in the serum of certain persons belonging to the group A_2B is well known (Landsteiner and Witt, 1926; Wiener, 1941; Wiener, 1943; Race, Taylor, Cappell and McFarlane, 1943). It was considered of interest therefore to ascertain whether the rabbit *alpha*$_1$ agglutinin likewise possessed this characteristic property. The three absorbed sera 788, 805 and 816 were therefore titrated at 5–6°, room temperature (18–21°) and at 37°, in parallel with natural *alpha*$_1$ agglutinin. The results are shown in Table IV. The thermal amplitude found for the naturally occurring *alpha*$_1$ agglutinin confirmed the observations of earlier workers, and showed that at 37° there was no significant agglutination, whereas at 5–6° the serum became to some extent unspecific in its action on the test-cells and, in the particular serum under test, agglutinated A_2 cells at a dilution as high as 1 : 32. The rabbit *alpha*$_1$ agglutinin remained quite specific for A_1 cells at 5°, and moreover showed no significant difference in the agglutination titre at the three temperatures investigated.

The neutralization of the rabbit *alpha*$_1$ agglutinin by the A-substance isolated from hog gastric mucin and from ovarian cyst fluid was investigated by means of the agglutination inhibition technique. Rabbit *alpha*$_1$ agglutinin (0·1 ml.) representing 2–3 completely agglutinating doses was mixed with 0·1 ml. of serial dilutions of the A-substances. After standing at room-temperature for 1 hour, 0·1 ml. of a 1 per cent suspension of A_1 cells was added, and the degree of agglutination was read after

the tests had stood for a further 2 hours at room temperature. The control tubes containing saline in place of A-substance showed complete agglutination, whereas those tubes containing either animal or human A-substance at dilutions between 1 : 200 and 1 : 200,000 showed no agglutination. The results indicate that A-substances inhibit the agglutinating action of rabbit *alpha*$_1$ agglutinin as completely as they are known to inhibit the common *alpha* agglutinin induced in rabbits by A-erythrocytes or the artificial A-antigens.

Attempts to absorb with sheep-cells *alpha*$_1$ agglutinin from absorbed rabbit serum were without success. Three natural *alpha*$_1$ sera obtained from persons belonging to group A_2B and three *alpha*$_1$ rabbit sera prepared by complete absorption with A_2 cells were treated with an equal volume of washed, packed sheep-cells. There was a fall in titre of one dilution only when the absorbed rabbit *alpha*$_1$ agglutinin was titrated against A_1 cells. The natural *alpha*$_1$ (human) serum showed no fall in agglutinins reactive with A_1 cells. The results of an examination of two anti-A rabbit sera after repeated absorption with sheep-cells are shown in Table V. The sera obtained from the rabbits before immunization showed no agglutination with sheep-cells at a dilution of 1 : 10, but, as will be seen from the results given in Table V, during immunization there was a marked production of sheep-cell agglutinin. The first absorption with sheep-cells removed the whole of the anti-sheep agglutinins, and left behind the greater part of the common *alpha* and *alpha*$_1$ agglutinins. Three further absorptions with sheep cells made no appreciable change in the titre against A_1 and A_2 cells. Sheep-cells were therefore only able to remove a small part of the agglutinins reactive against A_1 and A_2 cells.

Similarly, it was observed that the immune anti-A sera contained a potent sheep-cell haemolysin. The nature of the haemolysin was investigated as follows: After immunization with the artificial A-antigen a pooled specimen of the serum from rabbits 816 and 817 which showed a haemolytic titre of 1 : 3200 was exhausted of sheep-cell agglutinin by absorption with sheep-cells, and was titrated against A_1 and A_2 erythrocytes; the agglutination titre with A_1 cells was 1 : 1600 and with A_2 cells 1 : 800. The haemolytic titre of the normal serum samples obtained from rabbits 816 and 817 was 1 : 100 and less than 1 : 25 respectively. Serial dilutions (0·5 ml.) of the absorbed serum, covering the range 1 : 25 to 1 : 1600, were mixed with equal volumes of guinea-pig complement (1 : 10) and of sheep-cells (4 per cent). The tubes were incubated at 37° for 1 hour. No haemolysis was observed. A known haemolytic serum was included as a positive control. The results indicate that rabbit anti-A (*alpha* and *alpha*$_1$) serum, after the sheep-cell agglutinin has been completely removed, has no haemolytic action on sheep-cells.

The sheep-cell agglutinin was recovered from the sheep-cells used for absorption and examined for its action on A_1 and A_2 cells, and for its capacity to act as a sheep cell haemolysin and agglutinin. A potent anti-A rabbit serum induced by means of the artificial antigen was absorbed at room temperature with a third of its volume of washed sheep-cells. The cells were removed, thoroughly washed with ice-cold saline and resuspended in saline at 50–55° and centrifuged at this temperature. The supernatant fluid was tested for agglutinins against A_1 and A_2 erythrocytes and against sheep-cells and for sheep-cell haemolysin. The results are given in Table VI, and show that the recovered immune-body is capable of agglutinating A_1, A_2 and sheep erythrocytes and of causing, in the presence of complement, sheep-cell haemolysis.

An absorbed rabbit serum containing common *alpha* agglutinin but free from *alpha*$_1$ agglutinin and sheep-cell haemolysin and agglutinin was readily prepared by absorbing undiluted anti-A rabbit serum with sheep-cells and then with A_2 cells. The A_2 cells were recovered, washed thoroughly with ice-cold saline, suspended in

TABLE IV.—*The Titration of Absorbed Anti-A Immune-Sera and Natural* Alpha$_1$ *Serum at Different Temperatures with* A_1 *and* A_2 *Erythrocytes.*

Temperature.	Test Red cells	Dilution of natural *alpha*$_1$.						Dilution of serum No. 788.							Dilution of serum No. 805.						Dilution of serum No. 816.						
		1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	1:16	1:32	1:64	1:128
5–6°	A_1	4	4	3	3	2	1	3	3	3	2	2	1	tr.	3	3	2	2	2	1	3	3	3	2	2	1	tr.
	A_2	3	2	2	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
18–21°	A_1	3	2	2	1	0	0	4	4	3	3	3	2	1	3	3	2	2	1	1	3	3	3	2	2	1	1
	A_2	0	0	0	0	0	0	tr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
37°	A_1	0	0	0	0	0	0	4	3	3	2	1	1	0	3	3	2	2	2	1	3	3	3	2	1	1	0
	A_2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Degrees of agglutination: 4, massive agglutination; 3, 2, 1, 0 as in Table I.

TABLE V.—*The Agglutinin Titre of Anti-A Rabbit Serum after Absorption with Sheep-Cells.*

Specimen.	Test-cells.	Dilution of serum 816.							Dilution of serum No. 817.						
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
Unabsorbed	A_1	4	4	3	3	3	2	1	4	4	4	3	3	3	2
	A_2	3	3	3	3	2	1	0	3	3	3	3	2	2	1
	Sheep	3	3	2	1	0	0	0	3	3	2	1	0	0	0
1st absorption	A_1	4	4	3	3	2	1	0	4	4	3	3	3	2	1
	A_2	3	3	3	2	1	0	0	3	3	3	2	1	1	0
	Sheep	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd absorption	A_1	4	4	3	3	2	1	0	4	4	3	3	2	2	1
	A_2	3	3	2	1	1	0	0	3	3	3	2	1	0	0
	Sheep	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3rd absorption	A_1	4	4	3	3	2	1	0	4	3	3	3	2	2	1
	A_2	3	3	2	1	1	0	0	3	3	2	2	1	0	0
	Sheep	No agglutination at 1 : 2							No agglutination at 1 : 2						
4th absorption	A_1	4	3	3	3	2	1	0	4	3	3	3	2	1	1
	A_2	3	3	2	1	0	0	0	3	3	2	1	1	0	0
	Sheep	No agglutination at 1 : 2							No agglutination at 1 : 2						

Degrees of agglutination: 4, massive agglutination; 3, 2, 1, 0, as in Table I.

TABLE VI.—*The Examination of Immune-body Absorbed from an Anti-A Rabbit Serum by Sheep-Cells and Subsequently Recovered.*

Test cells for agglutination.	Dilution of immune-body recovered from sheep-cells.										
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	1 : 2048
A_1	3	3	3	3	3	3	3	3	3	2	1
A_2	3	3	3	3	3	3	3	2	1	0	0
Sheep	3	3	3	3	3	3	3	3	3	1	0
Haemolysis test	–	–	–	4	4	4	4	3	2	1	1

Degrees of agglutination as in Table I.
Degrees of haemolysis: 4, complete; 3, almost complete; 2, definite; 1, trace.

saline at 50–55° and centrifuged at this temperature. The supernatant fluid was titrated against A_1 and A_2 erythrocytes and sheep-cells. The results are shown in Table VII. Since it has already been shown that *alpha*$_1$ agglutinin is not absorbed by A_2 erythrocytes, it is evident that the immune-body recovered from the A_2 cells is common *alpha* agglutinin.

TABLE VII.—*The Examination of Immune-body Absorbed from an Anti-A Rabbit Serum by A_2 Cells and Subsequently Recovered.*

Test cells for agglutination.		Dilution of immune-body recovered from A_2 cells.								
No.	Type.	1 : 16	1 : 32	1 : 64	1 : 128	1 : 256	1 : 512	1 : 1024	1 : 2048	1 : 4096
1	A_1	4	4	4	4	3	3	2	1	0
2	A_1	4	4	4	3	3	2	2	1	0
3	A_1	4	4	4	3	3	3	2	1	0
4	A_2	4	3	2	2	1	1	0	0	0
5	A_2	3	3	2	2	2	1	1	0	0
6	A_2	3	3	3	2	2	2	1	0	0
7	Sheep	0	0	0	0	0	0	0	0	0

Degrees of agglutination as in Table V.

DISCUSSION.

An examination of a number of anti-A immune sera induced in the rabbit by means of an artificial antigenic complex built up from A-substance of human origin and the conjugated protein component derived from the O-somatic antigen of *Bact. shigae* has revealed the presence of at least three kinds of immune-body. The unabsorbed sera agglutinate A_1, A_2 and sheep erythrocytes and, in the presence of complement, cause the lysis of sheep-cells. Absorption of these sera with suitable quantities of A_1 erythrocytes removes all agglutinins for A_1 and A_2 erythrocytes and agglutinins and haemolysins for sheep-cells. Treatment of the sera with A_2 cells removes all agglutinins reactive with A_2 erythrocytes and sheep-cells and the sheep-cell haemolysin. The immune-body remaining reacts with A_1 erythrocytes only, and is therefore a specific reagent for the detection of these cells. Several of these specific reagents reactive against A_1 cells only have been prepared and employed for the detection of A_1 erythrocytes in a series of 75 A bloods. The A_1 bloods in the series were identified by means of a naturally occurring *alpha*$_1$ agglutinin obtained from a person of genotype A_2B. In no instance was there a disagreement between the reactions given by the absorbed rabbit immune-serum and the natural human *alpha*$_1$ agglutinin. It appears, therefore, that the absorbed serum prepared from rabbit anti-A agglutinin by absorption with A_2 erythrocytes is a trustworthy reagent for the detection of A_1 cells. The absorbed sera are easy to prepare, are considerably more potent than the *alpha*$_1$ agglutinin occurring naturally in human serum, and show no thermal amplitude. The rabbit *alpha*$_1$ agglutinin, like the human *alpha*$_1$ agglutinin, is not absorbed by sheep cells.

The anti-sheep cell agglutinin present in an anti-A rabbit immune-serum can be removed by absorption with sheep-cells. There remains an absorbed serum that reacts with A_1 and A_2 erythrocytes only. The sheep-cell agglutinin can be recovered from the sheep-cells employed for absorbing the sheep-cell agglutinin from the anti-A rabbit serum, and the recovered immune-body will be found to agglutinate A_1, A_2 and sheep-cells and to haemolyse sheep-cells in the presence of a complement. The production of an immune-body with these properties, as a direct result of immunizing rabbits with an antigen made from an A-substance of human origin, indicates that there is present in the human A-substance a specific structure that is closely related to a component of the sheep red-cell envelope.

After the removal of the sheep-cell agglutinin from an anti-A serum, treatment of the *alpha* and $alpha_1$ agglutinins which remain with A_2 cells removes the common *alpha* immune-body. The latter agglutinin can be recovered from the A_2 cells and the immune-body so obtained is found to agglutinate A_1 and A_2 cells, but not sheep-cells. The agglutinin is, therefore, common *alpha* agglutinin.

The demonstration and separation of at least three different kinds of antibody molecules—$alpha_1$ agglutinin reactive against A_1 cells only, *alpha* agglutinin reactive with A_1 and A_2 cells, and sheep-cell agglutinin acting on A_1, A_2 and sheep-cells—from an immune serum formed in the rabbit with an apparently homogeneous artificial antigen offers additional evidence for the belief that different types of immune-body can be induced by the different or overlapping determinant structures of the hapten component of the antigen.

SUMMARY.

1. Rabbit sera which agglutinate strongly erythrocytes of the phenotype A_1 and A_1B but not those belonging to the sub-groups A_2 and A_2B can be readily prepared by absorbing with A_2 cells anti-A rabbit sera that have been induced by means of an artificial antigen built up from A-substance of human origin and a conjugated protein component of bacterial somatic-antigen.

2. The action of the rabbit $alpha_1$ agglutinin on A_1 erythrocytes is completely inhibited by A-substance of human or animal (hog) origin. The $alpha_1$ agglutinin shows no significant thermal amplitude, and is not absorbed by sheep-cells.

3. An *alpha* agglutinin which agglutinates A_1 and A_2 cells, but not sheep-cells, can be isolated from rabbit anti-A serum.

4. The artificial A-antigen also induces the formation of a sheep-cell agglutinin and haemolysin. This immune-body, recovered from sheep-cells, agglutinates A_1 and A_2 erythrocytes.

REFERENCES.

Dungern, E. von, and Hirszfeld, L.—(1911) *Z. ImmunForsch.*, **8**, 526.
King, H. K., and Morgan, W. T. J.—(1944) *Biochem. J.*, **38**, (Proc.) x.
Landsteiner, K., and Witt, S.—(1926) *J. Immunol.*, **11**, 221.
Idem and Levine, P.—(1926) *Ibid.*, **21**, 441.—(1929) *Ibid.*, **17**, 1.
Morgan, W. T. J.—(1943) *Brit. J. exp. Path.*, **24**, 41.—(1944) *Brit. med. Bull.*, **2**, 165.
Idem and King, H. K.—(1943) *Biochem. J.*, **37**, 640.
Idem and van Heyningen, R.—(1944) *Brit. J. exp. Path.*, **25**, 5.
Idem and Watkins, W. M.—(1944) *Ibid.*, **25**, 221.
Race, R. R., Taylor, G. L., Cappell, D. F., and McFarlane, M. N.—(1943) *Brit. med. J.*, ii, 289.
Taylor, G. L., Race, R. R., Prior, A. M., and Ikin, E. W.—(1942) *Ibid.*, ii, 572.
Wiener, A. S.—(1941) *J. Immunol.*, **41**, 181.—(1943) 'Blood Groups and Transfusion,' Baltimore.

A NEW TEST FOR THE DETECTION OF WEAK AND " INCOMPLETE " RH AGGLUTININS.

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IN this paper the details will be presented of a method which has been developed to amplify the effect of weak (or subliminal) Rh agglutinins and " incomplete " antibodies, by the action, on the sensitized cells, of a rabbit anti-human-globulin serum. The test was originally intended as an instrument of research ; but it promises to be of practical use, since it has detected Rh immunization in some cases where the usual tests have failed to do so. A brief account of this method has already been published (Coombs, Mourant and Race, 1945).

It must be common experience, and not only our own, that numerous cases of suspected haemolytic disease of the newborn, and of reaction to blood transfusion, occur in which the history and Rh arrangement strongly suggest that immunization has taken place, but in which the crucial demonstration of Rh antibody is lacking. In another troublesome group of cases, the serum under investigation agglutinates only certain of the Rh positives in the panel of test cells, and these only weakly. Such weak reactions are liable to be confused with those due to non-specific agglutination, sometimes seen when a cell suspension is added to an undiluted " inert " serum. In these cases, if a fairly large panel of Rh positive and Rh negative cells is used, a statistical assessment of the distribution of the faint reactions may make the presence of anti-Rh agglutinin reasonably certain, but often the issue is still left in doubt. The results of tests for the " incomplete " antibody also may be doubtful. It would seem therefore that these routine tests demand a certain threshold concentration of agglutinin, or " incomplete " antibody, before the results are unequivocal. By the method to be described, red cells sensitized with weak antibodies can be strongly agglutinated. We have confined our work to the Rh system, but the principle may be found to have wider applications in serology, and is not without theoretical interest. Perhaps the most significant effect so far observed is the strong agglutination of cells sensitized by the " incomplete " antibody.

> The " incomplete " or " blocking " antibody (Race, 1944, and Wiener, 1944) differs from other Rh antibodies in that it combines with homologous antigen, but fails to agglutinate the cells carrying this antigen. Until recently its action could only be detected by the failure of " sensitized " or " blocked " cells to agglutinate when subsequently exposed to the equivalent " complete " agglutinin. This is the basis of the inhibition or " blocking " test.

Wiener has recently published a paper (1945) in which he also claims to have produced agglutination of cells sensitized with the " incomplete " antibody. From the following

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description by Wiener it will be seen that his method and ours depend on essentially different immunological phenomena:

> "The new technique of Rh testing differs from the standard method in the substitution of normal, compatible human plasma or serum in place of saline solution as a diluent in the tests. That is, all the blood suspensions are prepared in the usual manner and strength (2 per cent), except that serum or plasma is used as the diluent instead of saline. Moreover, the serum being examined for Rh antibodies is tested undiluted or, if it is tested in dilutions as in titrations, compatible serum or plasma is used as the diluent instead of saline. For reasons which will soon become evident, the name 'conglutination test' is proposed for the new test, in order to distinguish it from the common agglutination test."

While not deprecating Wiener's test in any way, one of us, who has been working on the conglutination test as an immunological method of diagnosis, and who has been carrying out research into its mechanism, finds it very difficult to accept Wiener's use of the name conglutination. Wiener says that he selected the name "because of the resemblance of the reactions to the so-called conglutinin (co-agglutinin) phenomenon which has been observed particularly in tests with bovine sera," and gives the following references (Muir and Browning, 1906; Bordet and Streng, 1909; Dean, 1911; Leschley, 1916). However, in each of the works referred to, the phenomenon of conglutination is shown to require the action of a "conglutinating" complement which is destroyed at a temperature of 56° C. for half an hour or even at a lower temperature. The factor in human serum which aids the agglutination of cells sensitized with the "incomplete" antibody in Wiener's test may be similar in character to conglutinin. However, conglutinin by itself has no observable action on cells, sensitized only with antibody, and which have not been exposed to a "conglutinating" complement. On the other hand, conglutinin with the aid of a "conglutinating" complement will cause massive clumping of cells sensitized with antibody. It must be admitted that a difference does exist in that Wiener uses the sera undiluted, which would be quite impracticable in ordinary conglutination tests. Wiener states that his test works equally well after the human serum used as the diluent has been heated at 60° C. for half an hour, which is certainly sufficient to destroy all the "conglutinating" complement. This seems to show quite definitely that the phenomenon is not one of conglutination in the original sense of the term (Bordet and Gay, 1906; Bordet and Gay, 1909; Lucas, Fitzgerald and Schorer, 1910; Bordet, 1939; Kagaya, Matuoka and Kanayama, 1940).

Dean (1911) defines these terms quite precisely:

> "1. *Agglutination:* By this is meant the well-known clumping of red cells by a specific antiserum or by a normal serum which possesses agglutinins for the red cells employed.
>
> "2. *Conglutination:* This is produced by the action of ox colloid (conglutinin) on cells which have been treated with homologous serum and with complement. In place of a serum prepared by the injection of an animal with red cells, a normal serum which contains a normal agglutinin for the red cells can, however, be employed.
>
> "3. *Co-agglutination:* An antigen and homologous antibody can under appropriate conditions agglutinate the red cells of another animal (preferably a guinea-pig)."

So far conglutinin has not been demonstrated in sera other than those of ruminants, but tests have not been carried out on undiluted sera.

It would seem that the phenomenon occurring in Wiener's test is more akin to that

recorded by Waaler (1939), who observed in human serum a factor which greatly increased specific agglutination of sheep cells by an immune serum. This substance was also thermostable to 60° C. for half an hour. It may also be similar to the " auxi-haemagglutinin " described by Manwaring (1906). Although the actual clumping of the cells in Wiener's test and in the conglutination phenomenon may be due to similar colloidal environments at the surface of the cells, the serological methods by which these conditions are brought about in the two cases are so totally different that it will inevitably lead to confusion in terminology if Wiener continues to describe his new test as a conglutination test.

Actually some months ago before Wiener announced his " conglutination " test for Rh sensitization, we had tried the conglutination test in the hope that it would detect cells sensitized with the " incomplete " antibody. Using four minimum sensitizing doses of equine " conglutinating " complement and four minimum sensitizing doses of bovine conglutinin we were unable to show conglutination of these cells. When the complement and the conglutinin were used much stronger, and when fresh bovine serum was used as a source of " conglutinating " complement and conglutinin, there was some indication of a reaction, but it proved so difficult to be sure that all the agglutinins to human cells had been completely absorbed out of these sera that the test was discarded. Especially difficult was the complete absorption of agglutinins from the " conglutinating " complement, which had to be done at 0° C., for here the situation was further complicated by the appearance of strong cold agglutinins. Human cells sensitized with the " incomplete " antibody, or with the Rh agglutinins which we examined, were found not to be sensitized to the action of guinea-pig or human complement *in vitro*. Neither could adsorption of guinea-pig haemolytic, or equine " conglutinating " complement by these sensitized cells be demonstrated. Finally the idea occurred to us, to see whether the antibody globulin on the sensitized cells would react specifically with a rabbit anti-human-globulin serum in some observable way, such as causing the cells to agglutinate. Strong agglutination of characteristic type was observed, with complete absence of agglutination of unsensitized cells. The application of the technique thus evolved constitutes the main subject of this paper.

METHODS.

Two drops of a 2 per cent suspension of washed blood cells of an appropriate Rh genotype are added to an equal volume of the serum under investigation in a 50 mm. × 7 mm. precipitin tube. The serum is usually used unheated, but may be heated at 56° C. for half an hour. Controls with serum known to contain no agglutinins for the cells, or with saline instead of serum, are put up at the same time. Glass caps 25 mm. × 11 mm. are placed over the tubes and the tubes then incubated at 37° C. for half an hour. Following this a small sample of the deposited cells is carefully withdrawn with a fine Pasteur pipette, placed on a glass slide and examined microscopically. The results at this stage should be the same as those found in the routine test for agglutinins. If agglutination is observed the test is carried no further. However the absence of agglutination may mean either that the serum contains no agglutinins for the cells used, or, on the other hand, that it contains an agglutinin which is too weak to be detected or is of the " incomplete " variety. To exclude these latter two possibilities the second stage of the test is carried out. The cells are washed three times with saline, to remove all human serum, and finally resuspended in two drops of saline to reconstitute a 2 per cent suspension. To this suspension is added an equal volume of an appropriate dilution of rabbit anti-human-globulin serum, which has previously been absorbed with a mixture of human A, B and O cells and

preferably also with cells of the type being used in the test, although this may be dispensed with in many cases as long as a control is set up to ensure that the rabbit antiserum at the dilution used contains no detectable agglutinins to the cells under test. After a further incubation of half to one hour at 37° C., by which time the cells will have settled, the tubes are re-examined macroscopically and microscopically for agglutination; though in practice the macroscopic reading has in every case proved sufficient.

If the blood cells have been sensitized in any degree with weak Rh antibody or "incomplete" antibody in the first stage of the test, the human globulin of the Rh antibody is presumably firmly attached at some points on their surface. The rabbit anti-human-globulin serum, reacting specifically with this "attached" globulin, causes the cells to agglutinate, if enough receptors are involved, while normal unsensitized cells are unaffected. Red blood cells possess an unusual surface (Abramson, Moyes and Gorin, 1942) in that they retain their own surface properties, even in the presence of large amounts of proteins. No protein of human serum is firmly adsorbed on to their surface unless it is of the nature of a specific antibody—and so only sensitized cells will be agglutinated by the rabbit serum.

To get the best results one or two points in the technique need special care. There seems to be an optimum cell concentration to aim at. If it is too light, the cells do not get the chance of forming compact agglutinated masses; a 2 per cent suspension has been found to be most satisfactory. After the first stage of the test the washing of the cells is important. If most of the human serum is not removed it will neutralize some of the rabbit anti-human-globulin serum, leaving perhaps insufficient antibody to react with the sensitized cells. For this purpose three washings were found to be desirable—although two often seemed to be sufficient. On the other hand, if more than three washings are given there is the theoretical possibility that some weakly attached agglutinin may be dissociated. Using this delicate test it has been found that, in the early stages great care is required in thoroughly rinsing the pipette in order to avoid carrying over traces of antisera from one tube to the next.

Rh Antigens and Antibodies: Fisher's Synthesis.

Since the Rh antigens and antibodies are to be discussed in some detail, it becomes necessary at this point to give some key to their complicated but very orderly interactions. Table I shows these interactions.

TABLE I.—*Rh Antibodies and Genes.*

Original names. Fisher's antigenic or genetic notation.			Genes.							
			Rh_1 CDe	Rh_2 cDE	rh cde	Rh_0 cDe	Rh′ Cde	Rh″ cdE	Rh_z CDE	Rh_y CdE
Antibodies.										
Wiener's recent names.	Fisher.	Cappell after Fisher.								
Anti-Rh′	Γ	anti-C	+	−	−	−	+	−	+	(+)
Anti-Rh_0	Δ	anti-D	+	+	−	+	−	−	+	(−)
Anti-Rh″	H	anti-E	−	+	−	−	−	+	+	(+)
	γ	anti-c	−	+	+	+	−	+	−	(−)
	∂	anti-d	(−)	(−)	(+)	(−)	(+)	(+)	(−)	(+)
	η	anti-e	+	−	+	+	+	−	(−)	(−)

Of 22 interactions predicted by Fisher 8 have been confirmed serologically. Those not yet confirmed are given in brackets.

Professor R. A. Fisher's synthesis of the results of the British work on the isolation of Rh genes was briefly described by Race (1944). Fisher, noticing the mutually antithetical reactions of two of the four distinct types of sera then known (Race, Taylor, Cappell and McFarlane, 1944), supposed that these two sera were reacting with allelic antigens. One of the antigens Fisher called C, and its allele c. The two remaining sera did not give antithetical reactions, so the antigens which they recognized were not allelic to each other though they presumably each had an allele. Instead of seven alleles of the Rh gene at one locus as had previously been supposed, Fisher pictured three adjacent loci each with two alleles, each allele corresponding to an antigen which was capable of reacting with one antibody only, as shown diagrammatically in Table II.

TABLE II.

Antibodies.	Loci on Chromosome.	Antibodies.
Γ	C or c	γ
Δ	D or d	δ
H	E or e	η

The hypothesis required two more antibodies, δ and η, unknown at that time. Mourant (1945) has found η, and has so far been able to confirm six of the reactions predicted for it. Altogether Fisher predicted 22 interactions. Eight of these have since been confirmed serologically. The remaining 14 not yet observed are shown in brackets in Table I.

The ordinary names for the Rh genes will be given throughout this paper, together with Fisher's antigenic or genetic formulae, without which the interactions are bewildering to anyone who is not thoroughly familiar with them. For naming the antisera, instead of Fisher's Γ, Δ, etc., Cappell's (1944) variation anti-C, anti-D, etc., will be used. It should perhaps be explained that in this paper when an antigenic formula gives C, c, D, E, or e, these have all been tested for directly by the five different sera each agglutinating one of them. On the other hand, d in a formula merely represents the absence of D from a chromosome, since we are not in the possession of an anti-d serum.

Choice of Rabbit Antisera.

The following rabbit antisera have proved to be reliable for this test after absorption with A, B and O cells:

1. Rabbit anti-human-pseudoglobulin.
2. Rabbit anti-human-globulin.
3. Rabbit anti-human-whole-serum.

Rh_2 rh cells $\left(\frac{cDE}{cde}\right)$ were sensitized with a serum containing the "incomplete" form of the antibody anti-D. After preliminary incubation the cells were washed three times with saline, and two drops of the final suspension were added to tubes containing an equal volume (two drops) of a serial dilution of the rabbit antiserum 1/4–1/64. The last tube in each row was a saline control. The results after further incubation are shown in Table III. All the sera were active up to a dilution of 1/64. Higher dilutions were not tested. The anti-pseudoglobulin serum was, however, weaker than the other two.

TABLE III.—*Agglutination of Rh_2 rh Cells $\left(\frac{cDE}{cde}\right)$ Sensitized with the "Incomplete" Form of the Antibody Anti-D and Exposed to the Following Antisera.*

Antisera	1/4.	1/8.	1/16.	1/32.	1/64.	Saline control.
Rabbit anti-human-pseudo-globulin :						
Macroscopic	+++	+++	++	++	++	–
Microscopic	++V	++V	++	+	+	–
Rabbit anti-human-globulin :						
Macroscopic	+++	+++	+++	++	++	–
Microscopic	++V	++V	++V	++V	++V	–
Rabbit anti-human-whole-serum :						
Macroscopic	+++	+++	++	++	++	–
Microscopic	++	++V	++V	++V	++V	–

KEY TO SYMBOLS.

Macroscopic readings +++, ++, +, – indicate degrees of agglutination.
Microscopic readings ++, +, (+), w, ?, – indicate degrees of agglutination.
V = Visual on the microscope slide.
R = Reading complicated by rouleaux formation.
Inc = "Incomplete."

In most of the tests up to date we have used the rabbit antisera diluted 1/8, but there seems no reason why a more dilute serum should not be used.

Failure of Normal Rabbit Serum to Cause Agglutination of Sensitized Cells.

Table IV shows that normal rabbit serum was found to have no action on sensitized cells. The serum of six normal rabbits was absorbed with A, B and O cells, and also with cells of the type being used in the test. These sera in a dilution of 1/4 did not cause agglutination of Rh_2 rh $\left(\frac{cDE}{cde}\right)$ cells sensitized with the "incomplete" form of the antibody anti-D, while rabbit anti-globulin-serum 1/8 caused these cells to agglutinate strongly.

TABLE IV.—*Failure of Normal Rabbit Sera to Cause Agglutination of Sensitized Cells.*

Cells	Genotype	Rh_2 rh	Rh_2 rh	Rh_2 rh	Rh_2 rh
	Rh antigens	$\frac{cDE}{cde}$	$\frac{cDE}{cde}$	$\frac{cDE}{cde}$	$\frac{cDE}{cde}$
Serum	Rh antibodies	Inc	..	Inc	..
		anti-D	—	anti-D	—
	Patient	Pr	Saline	Pr	Saline
Incubation :					
Macroscopic reading		—	—	—	—
Microscopic ,,		—	—	—	—
Washed and rabbit sera added :		Rabbit antiserum 1/8		Normal rabbit 1/4	
Macroscopic reading		+++	—	—	—
Microscopic ,,		++V	—	—	—

Tests using five other normal rabbits gave identical results.
For key to the symbols see Table III.

RESULTS.

The results obtained by the use of this new test can best be recorded in tabular form.

Detection of " Incomplete " Rh Antibodies.

Table V records the results of tests on sera known to contain " incomplete " Rh antibodies. Inhibition or " blocking " tests were carried out according to the method of Wiener (1944). Wiener's new " conglutination test " was also performed on each serum.

It will be noticed that our test detected sensitization by " incomplete " antibody in every case. Wiener's " conglutination test " worked well in the cases where the sera contained " incomplete " anti-D only, but for some unknown reason was not reliable where the sera contained anti-C as well as the " incomplete " anti-D. Nor did Wiener's test seem reliable in the detection of the " incomplete " from of anti-c in a serum which also contained the " complete " form of this antibody. This serum containing " incomplete " anti-c has been mentioned by Callender, Race and Paykoç (1945).

Difficulties were introduced into the reading of the results of Wiener's test by the marked rouleaux formation, due presumably to the high concentration of serum present. This is shown in Table V and Table VI.

Detection of Weak Rh Agglutinins.

Table VI shows the results of tests on sera, from cases very suggestive of immunization. By the ordinary test the agglutinins were so weak that they could hardly be detected, or were quite undetectable with the particular cells used. However, by carrying the test a stage further and adding rabbit antiserum the incompatibilities were well shown up.

Of special interest in this series is the serum Br, which is the only known serum at the present time containing the agglutinin anti-e or η (Mourant, 1945). This serum unfortunately is of such low titre that it is thought to give false negatives with some cells containing only a single dose of the antigen e. An instance of this is given in the table, where cells of the genotype Rh″ rh $\left(\frac{\text{cdE}}{\text{cde}}\right)$ failed to be agglutinated by the ordinary test, yet were shown by our new test to be sensitized.

To Show that each of the " Complete " Rh Agglutinins is able to Act as an Antigen for the Rabbit Antiserum.

Table VII records a series of tests on cells, sensitized by each of the known Rh agglutinins, to show that each of these agglutinins is able to act as a specific antigen for the rabbit antiserum. Most of these sera gave agglutination at the end of the first stage, and so normally the test would not have been continued. However, the cells were resuspended, washed, and exposed to rabbit antiserum, and in each case specific agglutination was obtained. That the rabbit antiserum contributed to the agglutination at this stage was shown by the macroscopic appearance of the deposit, which was similar to that caused by rabbit antiserum in cases where anti-Rh human serum alone had produced no visible effect.

It may be stated that a very old serum containing an anti-E agglutinin has been tested which gave specific agglutination of homologous cells by the ordinary test, and yet our test failed to detect sensitization. The reason for this is obscure. There would seem to be the possibility that in the ageing of the serum the globulin part of the antibody had become denatured and changed its specificity, or completely lost its antigenicity.

TABLE V.—*Detection of "Incomplete" Rh Antibodies.*

Cells	Genotype	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_1\ Rh_1}{CDe/CDe}$	$\frac{Rh_1\ Rh_1}{CDe/CDe}$	$\frac{Rh_1\ Rh_1}{CDe/CDe}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_2\ rh}{cDE/cde}$	$\frac{Rh_1\ rh}{CDe/cde}$
	Rh. antigens															
Serum	Rh antibodies	anti-C	..	anti-C	anti-C	anti-C	anti-C	anti-C	..	..	..	..	..	..	..	anti-c
		Inc	—	Inc	Inc	Inc	Inc	Inc	—	Inc	Inc	Inc	Inc	Inc	Inc	Inc
		anti-D	..	anti-D	anti-D	anti-D	anti-D	anti-D	..	anti-D	anti-D	anti-D	anti-D	anti-D	anti-D	anti-c
	Patient	PR	Saline	TI	BR	HO	BA	O46	RA	MO	PA	HA	MO	PA	HA	MOO
Incubation :			*						†							
Macroscopic reading		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Microscopic ,,		—	—	?	—	—	?	—	—	—	—	—	—	—	—	—
Washed and rabbit anti-glob. 1/8 added :																
Macroscopic reading		++	—	++	++	++	++	++	—	++	++	++	++	++	++	+++
Microscopic ,,		++V	—	++V	++V	++V	++V	++V	—	++VV	++VV	++V	++VV	++V	++V	++V
"Blocking test"		BL	..	BL	BL	BL	BL	BL	..	BL	BL	BL	BL	BL	BL	BL
Wiener's "conglutination test"		R	..	R	R	R	R	R	R	..	..	..	..	..	..	..
		w	..	+	?	w	?	?	?	++V	++V	++V	++V	++	++V	w

* Saline controls as this put up with each test—all gave no agglutination.

† Negative control.

Cells and sera in these tests are of course all compatible on the AB and O system.

(For key to the symbols see Table III.)

TABLE VI.—*Detection of Weak Rh Agglutinins.*

Cells: Genotype	Rh″ rh / cdE / cde	Rh″ rh / cdE / cde	Rh_2 rh / cDE / cde	Rh_1 Rh_2 / CDe / cDE	Rh_1 Rh_1 / CDe / CDe	Rh_2 rh / cDE / cde	Rh_1 Rh_2 / CDe / cDE	Rh_2 rh / cDE / cde	Rh_2 rh / cDE / cde	Rh_2 rh / cDE / cde
Cells: Rh. antigens										
Serum: Rh antibodies	anti-e	—	anti-e	anti-D	anti-D	anti-D	anti-D	anti-E	anti-D	?
Serum: Patient	BR	RA *	BR	BE	BE	BE	BE	CA	WH	SH
Incubation:										
Macroscopic reading	—	—	—	—	—	—	—	—	—	—
Microscopic ,,	—	—	w	+	—	—	—	w	—	—
Washed and rabbit anti-globulin 1/8 added:										
Macroscopic reading	+++	—	+	++	++	+++	++	+++	++	—
Microscopic ,,	++	—	++	++V	++V	++V	++V	++V	++V	—
"Blocking test"	..	—	..	—	—	—	—	—	?	—
Wiener's "conglutination test"	++	—	++V	++V	+	(+)	+	++V	$+^R$	$?^R$

* As in this case each type of cell was tested with a "heterologous" serum and found to give no agglutination by our new test. Also where possible each serum was tested with "heterologous" cells and found to be give no agglutination.

(For key to the symbols see Table III.)

TABLE VII.—*Ability of each of the "Complete" Rh Agglutinins to act as an Antigen for the Rabbit Antiserum.*

Cells: Genotype	Rh′ rh / Cde / cde	rh rh / cde / cde	Rh_2 rh / cDE / cde	rh rh / cde / cde	Rh_2 rh / cDE / cde	rh rh / cde / cde	Rh′ rh / Cde / cde	Rh_1 Rh_1 / CDe / CDe			rh rh / cde / cde	Rh_2 $Rh^"$ / cDE / cdE
Cells: Rh antigens												
Serum: Rh antibodies	anti-C	anti-C	anti-D	anti-D	anti-E	anti-E	anti-c	anti-c	anti-d	anti-d	anti-e	anti-e
Serum: Patient	PR	PR	RI	RI	MA	MA	CH	CH	.. *	..	BR	BR
Incubation:												
Macroscopic reading	—	—	—	—	—	—	+	—	..	..	—	—
Microscopic ,,	+	—	++	—	++	—	++	—	..	..	+	—
Washed and rabbit anti-globulin added:												
Macroscopic reading	+++	—	++	—	++	—	+++	—	..	..	+++	
Microscopic ,,	++	—	++	—	++	—	+	—	..	..	++V	

* The anti-d agglutinin has not yet been demonstrated.

(For key to the symbols see Table III.)

Observations on the Test.

The agglutination by the rabbit antiserum of cells sensitized with "incomplete" antibody seems stronger than that produced when the sensitization is by "complete" agglutinins.

If the agglutination by an Rh agglutinin in the first stage is very weak, the rabbit antiserum produces strong agglutination; while, on the other hand, if the agglutination in the first stage is very strong, in the second stage it tends to become weaker. It must be remembered, however, that if a good reaction is obtained by the Rh iso-agglutinin, there is no need to carry the test further.

The agglutination in the second stage can often be seen within a few minutes of adding the rabbit antiserum. This agglutination appears to be equally complete whether the reaction takes place at 37°C., 22° C., or even 8° C., though the sedimentation is slower at the lower temperatures.

The constant parallelism between the macroscopic and microscopic readings has already been mentioned.

DISCUSSION.

We have shown that cells sensitized with "incomplete" antibody are usually agglutinated more strongly by the rabbit antiserum than are cells sensitized with "complete" antibody. Further work along these lines may lead to some information as to the essential difference between these two types of antibody. For instance, it would be interesting to compare the agglutination of the variously sensitized cells by the sera of rabbits immunized against the different electrophoretic protein fractions of human serum.

Diamond and Abelson (1945) states that prolonged heating of a serum at 56° C. inactivates anti-Rh agglutinin. It was also stated by us (Coombs and Race, 1945) that heating a serum containing both "complete" anti-C agglutinin and "incomplete" anti-D at 65° C. for 10 minutes destroyed all detectable anti-C, while leaving the "incomplete" anti-D apparently unchanged in quality or quantity. It now seems that this statement is not quite correct, since although after such treatment the anti-C could no longer be detected by the usual agglutination test or by "blocking tests," or even by Wiener's "conglutination test," yet by the present test, cells containing C but not D were strongly agglutinated. Thus, although the heated anti-C could on longer cause agglutination, nor had acquired "blocking" properties, yet it was still capable of "sensitizing" appropriate cells.

The new test has usefully been applied to increase the effect of a weak but diagnostically significant serum. As above stated, only one serum (Br) has so far been found to contain the agglutinin η or anti-e (Mourant, 1945), and this is of low titre. It usually gives a well-defined agglutination with cells homozygous for the antigen e, but these are, in any case, always recognizable by being unagglutinated by anti-E. Cells containing only one dose of e tend, on the other hand, to give weak and occasional false negative reactions with the available serum, and so to be confused with cells not containing e. It is unfortunate that this is precisely the distinction which no antibody except anti-e is capable of demonstrating. The distinction is of particular importance in the case of fathers of babies with haemolytic disease, since fathers of genotype $Rh_2\ Rh_2\left(\frac{cDE}{cDE}\right)$ will have none but Rh-positive and probably affected babies, whereas men of genotype $Rh_2\ rh\left(\frac{cDE}{cde}\right)$ can also have Rh-negative unaffected children.

Certain cells of probable genotype Rh″ rh $\left(\frac{\text{cdE}}{\text{cde}}\right)$ which were not, however, directly agglutinated by this serum were investigated. Had they been truly η-negative they would have belonged to the very rare genotype Rh″ Rh″ $\left(\frac{\text{cdE}}{\text{cdE}}\right)$. The new test showed they had been sensitized by the serum and were therefore Rh″rh. Cells which were previously known to be of genotype Rh_2 Rh″ $\left(\frac{\text{cDE}}{\text{cdE}}\right)$, and which therefore contained no antigen e, were unagglutinated by the new test. If the enhancement of the action of this rare serum (Br.) is as consistent as it appears to be, the usefulness of the serum for genotyping will be greatly increased.

Using this test, other " incomplete " iso-agglutinins or weak agglutinins should be quickly brought to notice. Diamond and Abelson (1945) have recently called attention to the existence of " incomplete " forms of the antibodies anti-C, anti-E and anti-c, while in quite a different field Levine and Gilmore (1945) report an " incomplete " form of the anti-sheep cell agglutinin found in cases of infectious mononucleosis. It would seem that the principle of the test could be applied to a number of antigen-antibody systems where for some reason the sensitization cannot be observed by any characteristic reaction. By the use of the rabbit anti-human-globulin serum it should be possible to demonstrate the actual sensitization *in vivo* of the red blood cells of an infant suffering from haemolytic disease even when no free antibodies are demonstrable in its serum.

Any abnormal condition, in which the human red cells strongly adsorbed globulin on their surface, would presumably cause the cells to be agglutinated by the rabbit antisera, but, as stated above, red cells tend to retain their own surface properties even in the presence of large amounts of proteins (Abramson, Moyes and Gorin, 1942).

The specific reaction between cells sensitized with " incomplete " antibody and rabbit anti-human-globulin serum seems to be almost conclusive evidence that the " incomplete " antibody is, at least in part, human globulin. This and the fact of its specificity seems good evidence for its being classified as an antibody.

From a survey of the preceding tables it is clear that our new test is more reliable than is Wiener's " conglutination test " in our hands. On theoretical grounds, too, our test seems preferable as having a well-defined immunological basis.

In this paper strong emphasis has been laid upon the detection of weak antibodies, but it must be remembered that in most cases of haemolytic disease and transfusion reaction the Rh incompatibility is quite easily detected by the direct agglutination test or by the " blocking " test. It is only in those cases where the result of these is doubtful or negative that the new test need be carried out. Future experience will determine whether the new test can replace the " blocking " test for routine use.

SUMMARY.

A new test is described for the detection of " incomplete " or weak antibodies, with special reference to Rh antibodies. The principle of the test is the use of an anti-human-globulin serum which will agglutinate red cells which have been sensitized, and thus have antibody globulin adsorbed at some points on their surface.

We should like to express our thanks to Mrs. M. E. Adair, who prepared the rabbit antisera used in this work.

Addendum.

Since this paper has been written, a paper by Moreschi (*Zbl. Bakt.* **46**, 49) has come to the notice of the authors. Moreschi in 1908 recorded some experiments on the agglutination of rabbit red blood cells sensitized with a goat anti-rabbit-cell immune serum (which in itself was too weak to cause agglutination), by exposing these washed cells to the serum of a rabbit immunized against goat serum.

REFERENCES.

Abramson, H. A., Moyes, L. S., and Gorin, M. H.—(1942) ' Electrophoresis of Proteins and the Chemistry of Cell Surfaces.' Reinhold Publishing Corporation, N.Y.

Bordet, J.—(1939) ' Traité de l'immunité dans les maladies infectieuses.' Masson, Paris, 2nd Edition, p. 402.

Idem and Gay, F. P.—(1906) *Ann. Inst. Pasteur*, **20**, 467.—(1909) ' Studies in Immunity.' New York : Wiley & Co.

Bordet, J., and Streng, O.—(1909) *Zbl. Bakt.*, **49**, 260.

Callender, S., Race, R. R., and Paykoç, Z. V.—(1945) *Brit. med. J.*, ii, 83.

Cappell, D. F.—(1944) *Glasg. med. J.*, November, 125.

Coombs, R. R. A., Mourant, A. E., and Race, R. R.—(1945) *Lancet*, ii, 15.

Coombs, R. R. A., and Race, R. R.—(1945) *Nature*, **156**, 233.

Dean, H. R.—(1911) *Proc. Roy. Soc.*, B, **84**, 416.

Diamond, L. K., and Abelson, N. M.—(1945) *J. clin. Invest.*, **24**, 122.

Kagaya, Matuoka and Kanayama.—(1940) *Jap. J. med. Sci., VII, Social Medicine*, **3**, 150.

Leschley, W. Z.—(1916) *Z. ImmunForsch.*, **25**, 219.

Levine, P., and Gilmore, E. L.—(1945) *Science*, **101**, 411.

Lucas, W. P., Fitzgerald, J. G., and Schorer, E. H.—(1910) *J. Amer. med. Ass.*, **54**, 441.

Manwaring, W. H.—(1906) *J. infect. Dis.*, **3**, 225.

Mourant, A. E.—(1945) *Nature*, **155**, 542.

Muir, R., and Browning, C. H.—(1906) *J. Hyg., Camb.*, **6**, 20.

Race, R. R.—(1944) *Nature*, **153**, 771.

Idem, Taylor, G. L., Cappell, D. F., and McFarlane, M. N.—(1944) *Ibid.*, **153**, 52.

Waaler.—(1939) *Rep. Proc. Third Int. Congr. Microbiol.*, 777.

Wiener, A. S.—(1944) *Proc. Soc. exp. Biol., N.Y.*, **56**, 173.—(1945) *J. Lab. clin. Med.*, **30**, 662.

COMPARATIVE POTENCY OF THE MAMMARY TUMOUR AGENT OF MICE OF DIFFERENT GENETIC CONSTITUTION.

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PREVIOUS experiments (Dmochowski, 1944*a*) have shown that dried breast tumour tissue of the R III strain of mice gives rise to breast cancer in C_{57} × Strong A hybrid mice. It also induces a high incidence of breast cancer in the same hybrid mice after preliminary transmission through two other strains of mice, viz.: S × R III and S. Thus it is clear that the mammary tumour agent of a high cancer strain of mice can induce breast cancer in susceptible mice of an entirely different genetic constitution from that of the strain from which the agent originated.

In extension of these observations the present experiments were undertaken to ascertain whether the mammary tumour agent from other high cancer strains of mice can give rise to breast cancer in susceptible mice which differ in their genetic constitutions from that of the strains from which the mammary tumour agent was obtained.

METHODS.

C_{57} × C_3H low cancer strain hybrid mice, born and raised in the Laboratories, were employed in the experiments, as test mice as, in our experience, these hybrid mice are susceptible to the induction of breast cancer. The incidence of breast cancer in our C_3H breeding females is 78 per cent at an average age of 10 months, while no mammary cancer has as yet occurred in the C_{57} black females kept in our Laboratories.

C_{57} × Strong A hybrid mice (Dmochowski, 1944*a*) and Ax strain mice (that is Strong A high cancer strain female mice deprived of the mammary tumour agent by being fostered by C_{57} low cancer strain females) were also used as test mice in the present experiments.

R III, C_3H and Strong A breast tumour tissue was used as the source of the mammary tumour agent. Several tumours from each strain of mice were minced together and desiccated in the usual way (Dmochowski, 1944*a*). The tumour tissue was stored in the ice chest for a period of 3–5 weeks. Before use, the tissue was resuspended in distilled water in a dilution of 1 : 4 and injected subcutaneously into the mice. Each mouse was given, at each injection, 0·5 c.c. of the suspension containing approximately 0·125 g. of breast tumour tissue. The injections were given at weekly intervals. Each mouse received 12 injections comprising altogether 1·5 g. of breast tumour tissue (R III, C_3H or Strong A tumour tissue).

The C_{57} × C_3H hybrid mice when 4 weeks of age were marked individually and divided into four groups. The first group received dried R III tumour tissue, the second dried C_3H tumour tissue, and the third dried Strong A tumour tissue. The fourth group served as controls.

Ax mice when 4 weeks of age were marked individually and divided into three groups. Mice of the first group received dried R III breast tumour tissue suspension;

mice of the second group received dried C_3H breast tumour suspension, and the third group served as controls.

The $C_{57} \times$ Strong A hybrid mice when 4 weeks of age were divided into two groups. Mice of the first group were given the suspension of dried C_3H breast tumour tissue, and those of the second group served as controls.

Both experimental and control mice of the strains employed in the experiment were forcibly bred by removing each litter within 24 hours of birth. All mice were kept under similar conditions, and on a diet of " Rat-cake " cubes with an unlimited supply of tap water.

RESULTS.

The results of the experiment with all groups of mice are summarized in Table I.

TABLE I.

Strain of mice.	Number of mice.	Source of mammary tumour agent.	Number of tumours appearing monthly														Tumours.		Average tumour age in months.
			7	9	10	11	12	13	14	15	16	17	18	19	20	22	Number.	Percentage incidence.	
$C_{57} \times C_3H$	14	R III	–	1	1	1	3	1	1	2	–	1	1	–	–	–	12	85·7	13·2
$C_{57} \times C_3H$	17	C_3H	–	–	1	–	1	1	3	1	3	–	–	1	1	–	12	70·5	14·9
$C_{57} \times C_3H$	24	Str. A	–	–	–	–	–	3	–	3	5	1	1	–	–	1	14	58·3	15·8
$C_{57} \times$ A	24	C_3H	–	–	–	–	–	4	4	4	5	–	–	–	–	–	17	70·8	14·6
Ax	33	R III	1	–	–	–	–	2	1	3	3	4	8	2	–	–	24	72·7	16·2
Ax	14	C_3H	–	–	–	–	–	1	–	–	–	2	3	1	1	–	8	57·1	17·5

No tumours developed in any of the litter mate control females which were forcibly bred and which were not injected with tumour tissue. This again confirms our previous findings that few or no tumours develop in susceptible mice after oestrogenic stimulation if the mammary tumour agent is lacking. (Dmochowski, 1944*a*, 1944*b*; Dmochowski and Gye, 1944).

As can be seen from Table I, there is a difference between the tumour incidence induced in $C_{57} \times C_3H$ hybrid female mice by the dried R III, C_3H and Strong A tumour tissues. Strong A tumour tissue gives the lowest tumour incidence at the highest tumour age, while C_3H tumour tissue gives a lower incidence than R III tumour tissue. Dried R III tumour tissue induces the highest incidence of breast cancer in $C_{57} \times C_3H$ female mice at the lowest average tumour age.

Similar differences in the breast tumour incidence and average tumour age are produced by the R III and C_3H tumour tissues when injected into Ax mice. Dried R III tumour tissue gives a higher incidence of breast cancer at a lower tumour age than dried C_3H tumour tissue.

There is, however, a difference between the results obtained in $C_{57} \times C_3H$ hybrid females and Ax female mice. Dried R III tumour tissue induces a higher incidence of breast cancer at a lower average age when injected into $C_{57} \times C_3H$ hybrids than when injected into Ax mice.

As can be seen from Table I the C_3H mammary tumour agent also gives a high incidence of breast cancer in $C_{57} \times$ Strong A hybrid mice.

Thus breast cancer can be induced in $C_{57} \times$ Strong A female mice not only by the mammary tumour agent in Strong A high cancer strain but also by the agent in C_3H and R III high cancer strains which have an entirely different genetic constitution from that of the $C_{57} \times$ Strong A hybrids (Dmochowski, 1945).

Similarly breast cancer can be induced in $C_{57} \times C_3H$ female mice by the agent in C_3H tumour tissue, and also by the agent in R III and Strong A tumour tissue which are of a different genetic constitution from that of the C_{57} and C_3H strain. A high

incidence of breast cancer can also be induced in Ax strain females by the mammary tumour agent in R III and C_3H strains which have again a different genetic constitution from the Strong A strain.

It can be concluded that the mammary tumour agent of any of the high cancer strains of mice in our Laboratories can produce breast cancer in susceptible strains of mice, although their genetic constitution differs from that of the strain from which the agent originated.

There exists, however, a difference in the tumour-producing activity of dried tumour tissue of the R III, C_3H and Strong A high cancer strains of mice bred in our Laboratories. That of the R III strain is greater than that of the C_3H strain, and dried Strong A tumour tissue is the least potent.

These results are in agreement with a previous paper (Dmochowski, 1945) in which the difference between the tumour producing potency of R III and Strong A agent was first described. They also show that the time of tumour appearance and the tumour incidence depend not only on the variable potency of the dried mammary tumour tissue, but also on the genetic constitution of the strain which receives the agent (C_{57} × Strong A and C_{57} × C_3H hybrid mice as compared with mice of the Ax strain).

Experiments are in progress to ascertain whether this variation in the potency of the dried mammary tumour tissues of mice of different genetic constitution is the result of differences in the potency of the agent they contain or whether it is dependent upon varying amounts of the agent present in tumours of these strains.

SUMMARY.

The present experiments have demonstrated that (1) dried tumour tissue of R III strain induces breast tumours in C_{57} × C_3H hybrids and Ax mice. (2) That of C_3H strain in C_{57} × C_3H and C_{57} × Strong A hybrids and Ax mice. (3) That of Strong A strain in C_{57} × C_3H hybrids. That is, the genetic constitution of the mice which developed cancer differed in each case from that of the strain which provided the mammary tumour agent.

Differences between the tumour producing activity of the dried R III, C_3H and Strong A tumour tissues have been shown.

The tumour producing potency of the dried R III tumour tissue (as shown by the breast tumour incidence and the average tumour age) is greater than that of dried C_3H tumour tissue, while dried Strong A tumour tissue is the least potent.

It remains undetermined whether the potency of the agent varies in different strains or whether tumours of different strains contain varying amounts of the same agent.

REFERENCES.

DMOCHOWSKI, L.—(1944*a*) *Brit. J. exp. Path.*, **25**, 138.—(1944*b*) *Ibid.*, **25**, 119.—(1945) *Ibid.*, **26**, 192.

Idem AND GYE, W. E.—(1944) *Ibid.*, **25**, 115.

EXPERIMENTAL NODULAR GOITRE.

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In a previous communication (Bielschowsky, 1944*a*) the production of benign and malignant tumours of the thyroid gland by the simultaneous feeding of 2-acetyl-amino-fluorene and allyl-thiourea has been reported. It was shown that the goitrous gland was susceptible to the action of a carcinogen which did not affect the unstimulated thyroid. I have now studied the effects produced by the successive feeding of 2-acetyl-amino-fluorene and allyl-thiourea on the thyroid gland of rats. The purpose of these experiments was to obtain material more amenable to analysis of the pathogenesis of the acetyl-amino-fluorene induced tumours of the thyroid, and to decide whether the simultaneous feeding of the carcinogenic and the goitrogenic substances was a requisite for the production of these neoplasms.

The question of whether simultaneous feeding necessarily implied combined action, could not be answered by the previous experiment. The interpretation was made still more difficult by the presence of cancers in organs other than the thyroid. Since malignant tumours can profoundly influence enzyme systems of distant organs, a possible effect of the other acetyl-amino-fluorene induced cancers on the thyroid had to be considered. Still another possible source of error had to be excluded ; it was necessary to find whether the acetyl-amino-fluorene was stored in the body of the rat after prolonged feeding. No evidence for such a storage could be obtained (Bielschowsky, 1945).

While these experiments were in progress, two important papers have been published which furnish valuable information. Van Dyke (1944) described the presence of adenomata of the thyroid in very old rats, and Griesbach, Kennedy and Purves (1945) reported the production of tumours of the thyroid by prolonged feeding of a Brassica seed diet.

METHODS.

Two different strains of rats were used, our inbred strain of Wistar rats and a strain of piebald rats imported two years ago, the latter being the offspring of three generations of brother-sister mating. The diet was the same as used in previous experiments (Bielschowsky, 1944) to which allyl-thiourea was added to supply daily 8 mg. per rat, or half this dose of acetyl-amino-fluorene.

All thyroids were cut in serial sections in order that not even minute adenomata might be overlooked.

RESULTS.

Group A : 5 young female albino rats received daily for 23 weeks 4 mg. of 2-acetyl-amino-fluorene, and for 2 further weeks half this amount. Then the carcinogen was replaced by 8 mg. of allyl-thiourea daily until the animals were killed. Multiple nodules numbering 15–20 were found in the thyroid of each of the three rats which received allyl-thiourea for 10 or more weeks.

Groups B and C served as controls for this experiment. In Group B the effect of allyl-thiourea on the thyroid of young normal females was studied, and in group C on the thyroid of animals of the same sex and age, inoculated with a slow-growing transplantable breast tumour. This tumour had originated in a female rat of the same strain after treatment with acetyl-amino-fluorene and the third generation of it was used. Histological study of the thyroids of these 11 animals revealed that four of them contained a single, minute adenoma, the earliest of which was seen after 8 weeks of stimulation. These single adenomata were situated in different parts of the gland, two arose in the centre and the other two near a pole. The presence of an actively growing transplanted tumour in the flank did not seem to have any influence on the development of the single nodules, nor did the duration of the stimulation have an effect on the frequency with which they arose. (Table I.)

TABLE I.

Group.	Rat No.	Weeks of stimulation (allyl-thiourea).	Thyroid.	Cancers in other organs.
A	3	2	0	Small intestine, duct. acust. ext.
	1	9	0	,, ,, breast.
	2	10	+++	Breast.
	5	11	+++	,, liver.
	4	14	+++	,, ,,
B	2	9	+	..
	5	10	0	..
	4	11	0	..
	3	13	0	..
	1	14	+	..
C	4	6	0	Transplanted breast tumour.
	5	8	+	,, ,, ,,
	3	10	0	,, ,, ,,
	6	11	0	,, ,, ,,
	2	12	+	,, ,, ,,
	1	12	0	,, ,, ,,

In Groups D and E the period of feeding of the carcinogenic agent was reduced to 15 weeks in the hope that the animals would develop malignant tumours at a later date than in experiment A, so that more time for feeding the goitrogenous substance would be available. For these experiments 5 young female piebald rats and 5 male albinos of the same age were chosen in order to obtain some information about the influence of sex and strain. As Table II shows, the results were in accordance with those obtained in Experiment A. In the thyroids of both groups multiple adenomata were found in all cases which developed malignant tumours in organs other than the thyroid and which received allyl-thiourea for 10 or more weeks.

As controls, piebald rats of the same age were used, receiving allyl-thiourea only. No nodules were found in the thyroids of these rats after 10 to 13 weeks of feeding the goitrogenous agent (Group F).

In order to learn more about the nature and fate of the single nodules seen in our strain of Wistar rats (groups B and C) 10 albino rats (5 males, 5 females) were given a

TABLE II.

Group.	Rat No.	Weeks of stimulation (allyl-thiourea).	Thyroid.	Cancers in other organs.
D	2	10	+++	Small intestine, duct. acust. ext.
	4	11	+++	Duct. acust. ext.
	1	12	0	None.
	3	16	+++	Duct. acust. ext.
	5	18	++	Liver (microsc.).
E	1	9	+	Liver (miscrosc.).
	4	12	++	,,
	5	14	++	,,
	3	16	+++	,,
	2	18	+++	,,
F	5	10	0	..
	2	10	0	..
	4	13	0	..
	3	15	0	..
	1	17	0	..
H (Methyl-cholanthrene)	4	10	0	..
	3	15	0	..
	2	16	0	..
	5	21	+	..
	1	25	0	..

diet containing allyl-thiourea. For this experiment rats born to and nursed by a mother bearing a cancer of the breast induced by acetyl-amino-fluorene were chosen in the hope that these animals might be more liable to develop neoplasms. The females received allyl-thiourea for 19, the males for 25 weeks. One female and two males were killed on the day the allyl-thiourea was withdrawn, the rest 2 to 8 weeks later. Table III gives the results of this experiment. Single adenomata were found in the thyroids of 5 rats ; their appearance varied according to the date on which the animals were killed. The nodules found in the thyroids of animals sacrificed while they were still receiving allyl-thiourea, showed the same picture as those in Group B or C. But the nodules present in rats killed after the withdrawal of the goitre-producing substance had a different structure and were classified as cyst-adenomata. This experiment supports the view that the single nodules seen in rats receiving allyl-thiourea only were true neoplasms, which did not disappear when the stimulation ceased. Four of

DESCRIPTION OF PLATES.

FIG. 1.—× 40. Single nodule (Group B) during stimulation with allyl-thiourea.

FIG. 2.—× 400. Same nodule as in Fig. 1.

FIG. 3.—× 40. Single nodule (Group G) 4 weeks after withdrawal of allyl-thiourea.

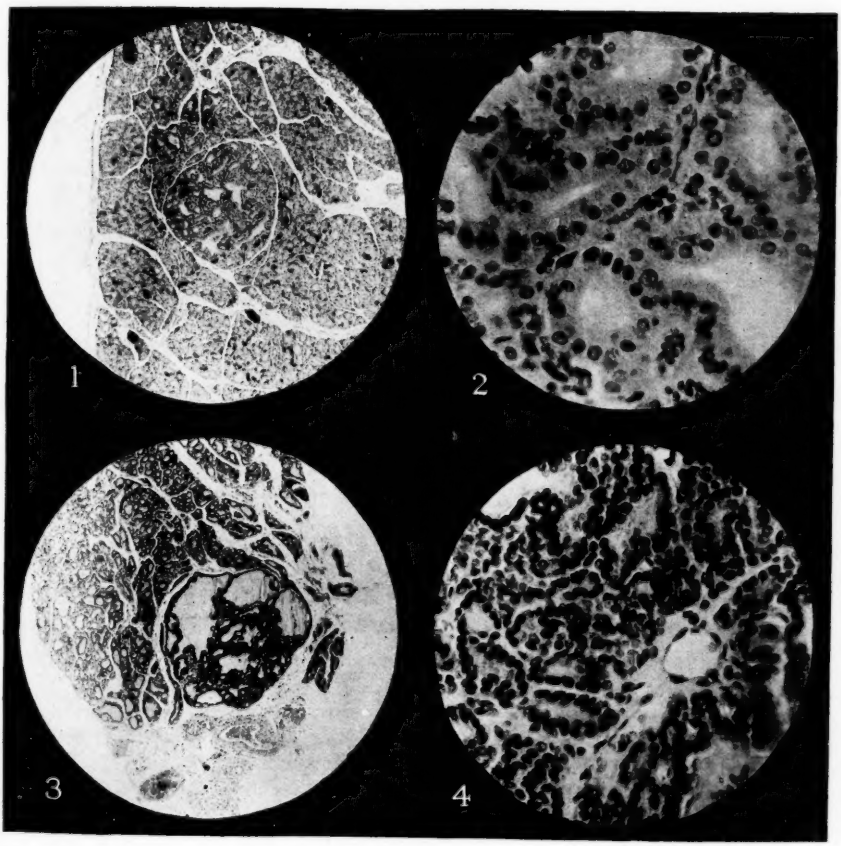
FIG. 4.—× 400. Same nodule as in Fig. 3.

FIG. 5.—× 40. Multiple nodules (Group A).

FIG. 6.—× 400. Detail from Fig. 5.

FIG. 7.—× 78. Nodule with tubular structure (Group D).

FIG. 8.—× 78. Thickening of the capsule (Group H).



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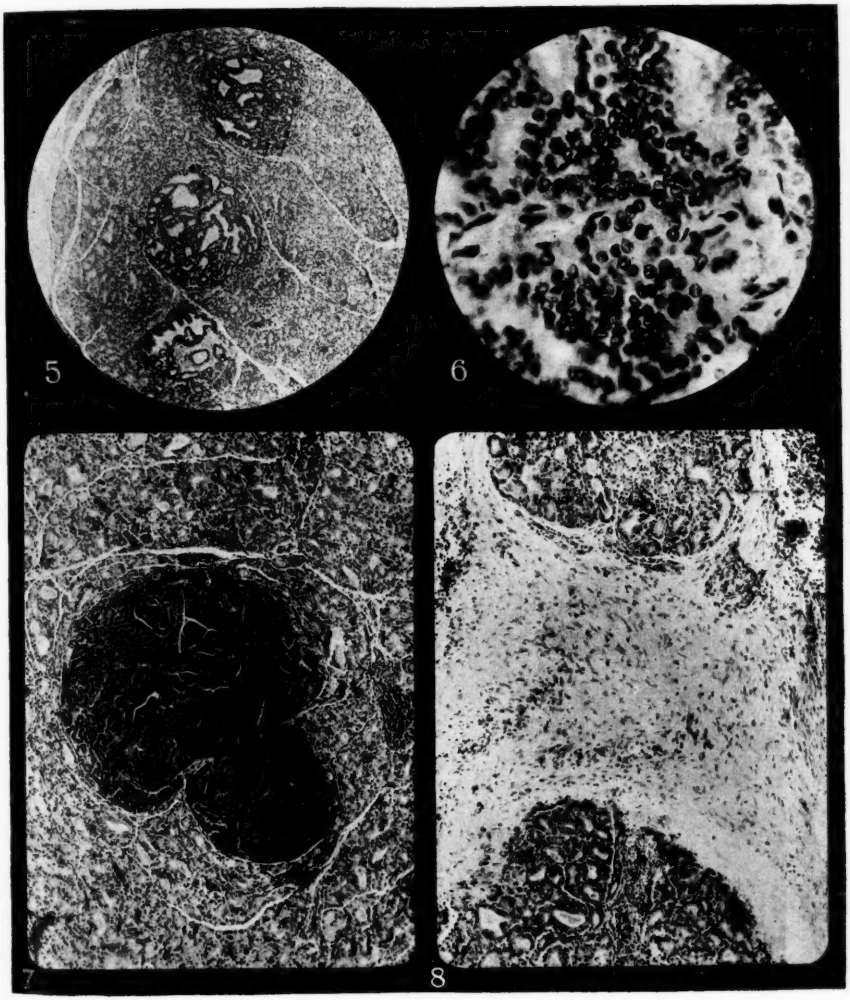

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TABLE III.

Group.	Rat No.	Weeks of stimulation (allyl-thiourea).	Duration of experiment.	Thyroid.
G (males)	1	25	25 weeks	+
	2	25	31 ,,	0
	3	25	31 ,,	+
	4	25	31 ,,	0
	5	25	25 ,,	0
G (females)	8	19	19 ,,	+
	10	19	21 ,,	0
	7	19	23 ,,	+
	9	19	25 ,,	+
	6	19	27 ,,	0

Tables I–III give the frequency of the adenomata of the thyroid: 0 no adenoma, + single adenoma, ++ 4–6 adenomata, +++ 7–20 adenomata.

the five nodules in group G were situated near a pole of the gland and only one was found in the centre.

Finally, in order to investigate if another carcinogen acted in a way similar to acetyl-amino-fluorene on the stimulated thyroid, 5 young female albino rats were painted three times weekly with a 0·3 per cent solution of methyl-cholanthrene in acetone for various periods. After 7 weeks of painting, allyl-thiourea was added to their diet. To avoid the development of tumours of the skin the methyl-cholanthrene was applied to a different area each time, so that during the whole experiment the same area was only painted twice. Only one minute nodule was found in the thyroid of one of these rats, but in all five there was a very marked thickening of the capsule of the thyroid, especially pronounced near the poles (Group H).

The histological picture of the single nodules seen in the controls was not very different from that of many present in the thyroids of the experimental rats. The great majority of these were made up of high cylindrical epithelium, the protoplasm of which stained a shade more intensely than normal with eosin. Their nuclei were richer in chromatin and stained deeper with haematoxylin than the nuclei of the surrounding tissue. The follicular structure was preserved in many instances. In the thyroids of the animals previously treated with acetyl-amino-fluorene, the cells forming the nodules tended to be more closely packed and mitoses to be more frequent than in the controls. The result was a less orderly arrangement. In some thyroids of the experimental group there were nodules which differed more; here the follicular structure had given way to a more tubular arrangement and the basophily of the nuclei was still more pronounced (Fig. 7). In no instance, however, had a nodule progressed to the state of malignancy. Whereas in the previously reported experiment (Bielschowsky, 1944*a*) three out of five female rats receiving the carcinogen and 6 mg. of allyl-thiourea simultaneously, developed lesions which bore the histological signs of early or advanced malignancy, after successive feeding of the two agents not a single nodule was found, where infiltration of the neighbourhood or the character of the cells would allow a diagnosis of malignancy.

Experiment G was planned to investigate the fate of the single nodules arising in the thyroids of albino rats after stimulation with the goitrogenous agent. It was found that after the withdrawal of the allyl-thiourea, the glands contained a relative

large single nodule, the epithelium of which had still the same staining qualities as seen during stimulation. But the epithelium was now low cuboid like that in the rest of the gland, and it lined large cysts, filled with densely stained colloid, into which it grew in a papillary fashion. Since these cyst-adenomata were single, like the more solid nodules seen during the period of stimulation, there could be no doubt that the nodules found in the control rats were benign tumours. They still followed the pattern of Marine's cycle to a remarkable degree. During stimulation the epithelium was high, during involution it was low. At variance with these cyclic changes of the epithelium was the occurrence of colloid in the single as well as in the multiple nodules during the period of stimulation. Many of the adenomata contained large amounts of colloid when the surrounding tissue contained little or none. The explanation must be that the neoplastic cell-groups were not under the control of the thyrotropic hormone to the same extent. Whereas the shape and size of the normal and of the neoplastic cells was equally influenced by the hormone of the pituitary, the neoplastic cells secreted appreciable amounts of colloid at a time when the rest of the gland contained little. In this respect they showed a low degree of autonomy compatible with the conception that they were benign tumours (Table III).

DISCUSSION.

The main result obtained in the experiments presented in this paper is the production of nodular goitres in rats who received first an effective dosage of acetyl-amino-fluorene and then allyl-thiourea. Whereas careful examination of the controls revealed that the thyroids of some animals contained a single adenoma, all rats but one of the experimental series, independently of strain or sex, developed multiple adenomata when the stimulation lasted for more than ten weeks.

Van Dyke (1944) studying the fate of the ultimo-branchial body in the rat found single adenomata in very old animals. He believed that these tumours take origin from cells derived from the epithelium of ultimo-branchial cysts. Our material is not suited to confirm this hypothesis ; a search for epithelial cysts lined with stratified squamous epithelium remained unsuccessful. This is not surprising, as according to van Dyke " the very existence of such cysts is usually incompatible with experimentally induced overactivity." It can only be stated that neither the single nor the multiple adenomata were found more frequently in the region where cysts derived from the ultimo branchial tissue would be expected to lie. But independent of the question from which cell element the benign tumours of the thyroid arise, it remains of importance, that as van Dyke discovered, they can occur in aged rats. It is worthy of note, however, that they rarely become malignant.

Recently Griesbach, Kennedy and Purves (1945) described the production of multiple adenomata of the thyroid by prolonged feeding of a diet containing 45 per cent Brassica seeds. They considered that these benign tumours were "due to the long-continued stimulation by the thyrotropic hormone and not to any specific neoplasia inducing effect of substances in the rape seed." Their experiments show that in a very susceptible strain of rats, hormonal stimulation maintained for many months produces results similar to the ones obtained in our rats in about the same number of weeks after previous treatment with acetyl-amino-fluorene. The role of the carcinogen is most obvious in the experiment where piebald rats were used (groups D and F), a strain less liable to react to thyrotropic hormone with the formation of adenomata. In the previous communication I expressed the opinion that " it is possible that the carcinogenic agent only hastens the process which may come about in a hyperplastic

gland spontaneously." The work of the New Zealand Group and the experiments presented in this paper make it very probable that this view is correct.

The question of the role of acetyl-amino-fluorene in the development of malignant tumours in thyroids stimulated by allyl-thiourea, when both drugs are fed simultaneously, remains unanswered. The absence of malignant tumours in the thyroids of rats who received first the carcinogenic and later the goitrogenic compound might be due to the relatively short time of stimulation obtainable in these experiments. But other explanations, like the one advanced by Mottram (1945) for the change of warts from benign to malignant, are possible. New experiments are in progress which aim at deciding this question.

SUMMARY.

The production of nodular goitres in rats by the successive feeding of 2-acetyl-amino-fluorene and of allyl-thiourea is described.

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REFERENCES.

Bielschowsky, F.—(1944) *Brit. J. exp. Path.*, **25**, 1.—(1944*a*) *Ibid.*, **25**, 90.—(1945) *Biochem. J.*, in press.

Griesbach, W. E., Kennedy, T. H., and Purves, H. D.—(1945) *Brit. J. exp. Path.*, **26**, 18.

Mottram, J. C.—(1945) *Ibid.*, **26**, 1.

Van Dyke, J. H.—(1944) *Anat. Rec.*, **88**, 369.